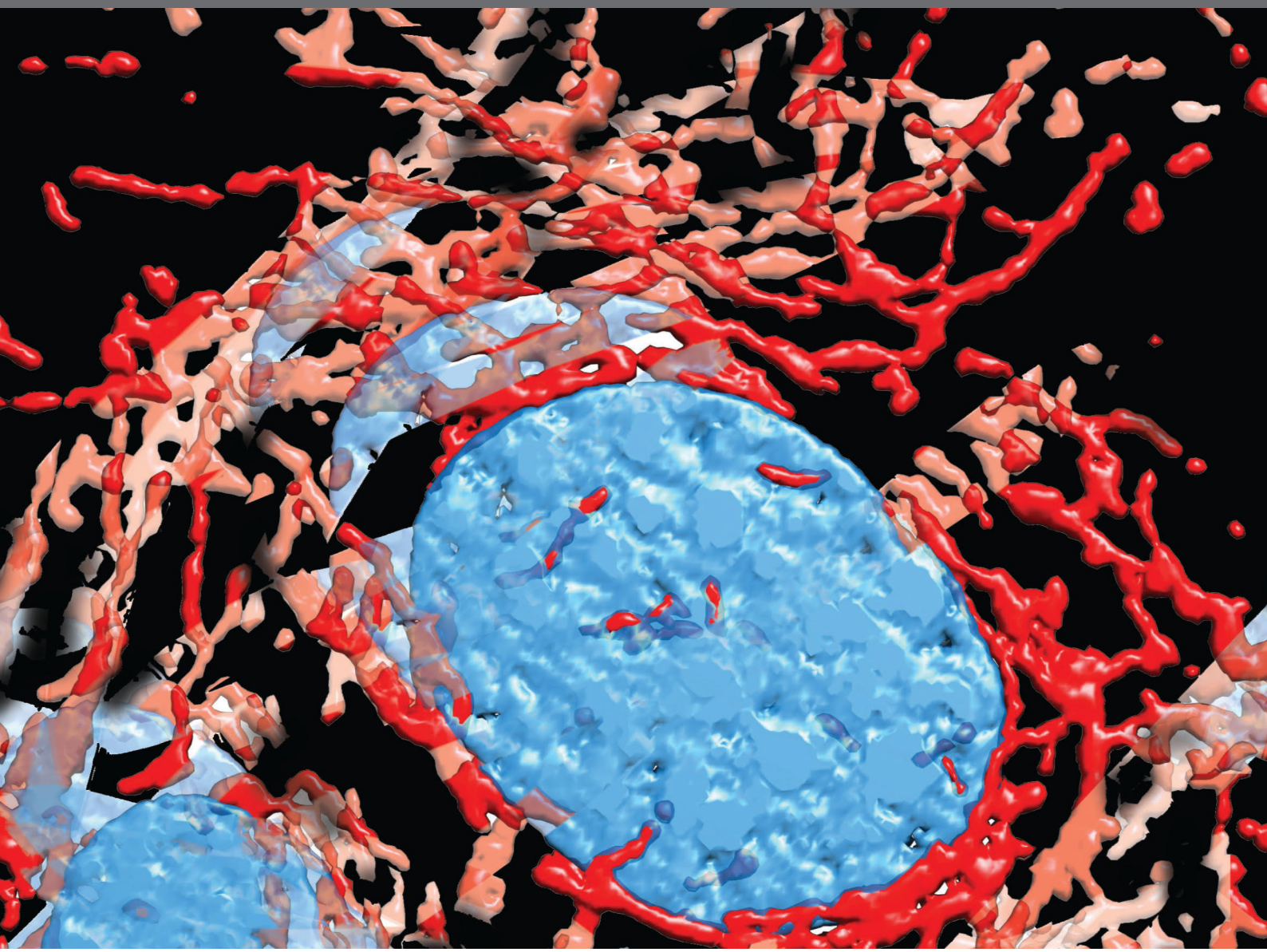
REDOX AND METABOLIC CIRCUITS IN CANCER

EDITED BY: Salvatore Rizza, Andrea Rasola, Danyelle M. Townsend and Giuseppe Filomeni

PUBLISHED IN: Frontiers in Oncology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88945-635-2
DOI 10.3389/978-2-88945-635-2

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

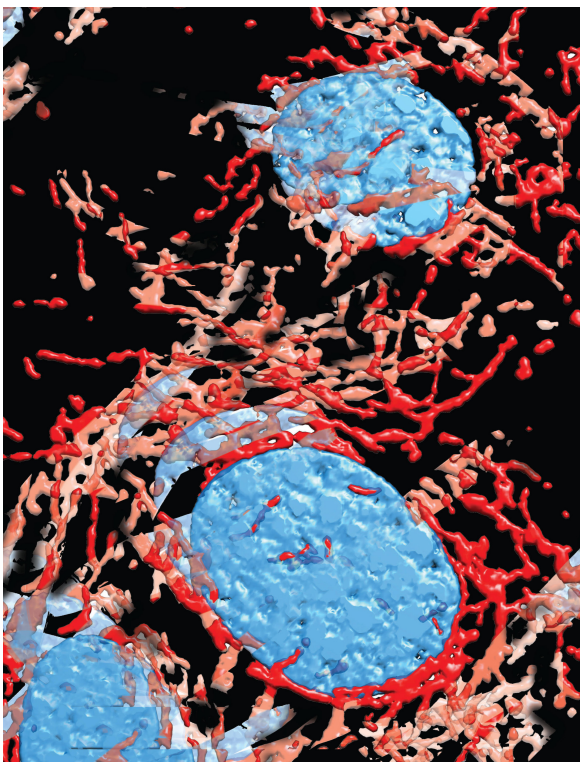
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

REDOX AND METABOLIC CIRCUITS IN CANCER

Topic Editors:
Salvatore Rizza, Danish Cancer Society Research Center, Denmark
Andrea Rasola, Università degli Studi di Padova, Italy
Danyelle M. Townsend, Medical University of South Carolina, United States
Giuseppe Filomeni, Danish Cancer Society Research Center, Denmark; University of Rome Tor Vergata, Italy



"Dynamic vision of cancer cell mitochondria"
Image: Salvatore Rizza

Living cells require a constant supply of energy for the orchestration of a variety of biological processes in fluctuating environmental conditions. In heterotrophic organisms, energy mainly derives from the oxidation of carbohydrates and lipids, whose chemical bonds breakdown allows electrons to generate ATP and to provide reducing equivalents needed to restore the antioxidant systems and prevent from damage induced by reactive oxygen and nitric oxide (NO)-derived species (ROS and RNS).

Studies of the last two decades have highlighted that cancer cells reprogram the metabolic circuitries in order to sustain their high growth rate, invade other tissues, and escape death. Therefore, this broad metabolic reorganization is mandatory

for neoplastic growth, allowing the generation of adequate amounts of ATP and metabolites, as well as the optimization of redox homeostasis in the changeable environmental conditions of the tumor mass. Among these, ROS, as well as NO and RNS, which are produced at high extent in the tumor microenvironment or intracellularly, have been demonstrated acting as positive modulators of cell growth and frequently associated with malignant phenotype. Metabolic changes are also emerging as primary drivers of neoplastic onset and growth, and alterations of mitochondrial metabolism and homeostasis are emerging as pivotal in driving tumorigenesis.

Targeting the metabolic rewiring, as well as affecting the balance between production and scavenging of ROS and NO-derived species, which underpin cancer growth, opens the possibility of finding selective and effective anti-neoplastic approaches, and new compounds affecting metabolic and/or redox adaptation of cancer cells are emerging as promising chemotherapeutic tools.

In this Research Topic we have elaborated on all these aspects and provided our contribution to this increasingly growing field of research with new results, opinions and general overviews about the extraordinary plasticity of cancer cells to change metabolism and redox homeostasis in order to overcome the adverse conditions and sustain their "individualistic" behavior under a teleonomic viewpoint.

Citation: Rizza, S., Rasola, A., Townsend, D. M., Filomeni, G., eds (2018). Redox and Metabolic Circuits in Cancer. Lausanne: Frontiers Media.
doi: 10.3389/978-2-88945-635-2

Table of Contents

5. REDOX AND METABOLIC ADAPTATION OF CANCER CELLS

6. BENCH TO BEDSIDE TRANSLATION

frontiers in Oncology

EDITORIAL
published: 26 September 2018
doi: 10.3389/fonc.2018.00403

Editorial: Redox and Metabolic Circuits in Cancer

Salvatore Rizza[1], *Andrea Rasola*[2], *Danyelle M. Townsend*[3] *and Giuseppe Filomeni*[1,4]*

[1] Redox Signaling and Oxidative Stress Research Group, Cell Stress and Survival Unit, Center for Autophagy, Recycling and Disease, Danish Cancer Society Research Center, Copenhagen, Denmark, [2] Department of Biomedical Sciences, University of Padova, Padova, Italy, [3] Department of Drug Discovery and Pharmaceutical Sciences, Medical University of South Carolina, Charleston, SC, United States, [4] Department of Biology, University of Rome Tor Vergata, Rome, Italy

Keywords: cancer metabolism, redox signaling, tumorigenesis, cancer progression, reactive oxygen species

Editorial on the Research Topic

Redox and Metabolic Circuits in Cancer

Biological processes in living cells require a constant supply of energy that primarily derives from the oxidation of biomolecules such as carbohydrates, proteins and lipids. The catabolism of biomolecules relocates electrons to the redox couples NAD^+/NADH, $NADP^+$/NADPH and FAD/FADH2, which represent the principal cofactors of dehydrogenases and reductases used by cells to sustain all endergonic process. While the couple $NADP^+$/NADPH is crucial for the antioxidant response and anabolic metabolism, NAD^+/NADH and FAD/FADH2 convey electrons to the mitochondrial transport chain resulting in the oxygen-dependent production of ATP, the energetic molecule sustaining the activity of the majority of cellular processes. As predictable, there are tight connections between metabolic fluxes, redox balance, oxygen availability, mitochondria function, and turnover.

A peculiar feature of living cells is their extraordinary adaptability to fluctuations in nutrient availability and environmental conditions due to the high plasticity of their biochemical machinery. The back side of the coin emerges, however, in pathologic settings. For instance, cancer cells reprogram the metabolic circuitries in order to sustain their high proliferation rate, invade other tissues, and evade death. An extensive reorganization of cell metabolism is, indeed, a pre-requisite for neoplastic transformation and facilitates tumor progression and metastasis. Cancer cells need to increase the levels of the molecular building blocks for membranes, nucleic acids, and proteins biosynthesis and, at the same time, need to produce elevate levels of ATP to sustain cell proliferation. This metabolic rearrangement, as well as exposure of cancer cells to diverse extracellular environments, inexorably results in the increase of reactive oxygen and nitrogen species (ROS and RNS, respectively), which act as positive modulators of cell growth and are frequently associated with malignant phenotype. The antioxidant capacity of cancer cells, as a consequence, readapts in order to tolerate the increased nitro-oxidative stress, this aspect having profound effects on chemoresistance to drugs. Metabolic rewiring, thus, generates cells that are able to face the adverse conditions they encounter in the process of tumor growth, such as nutrient paucity and nitroxidative stress or anticancer therapies.

The study of the intimate connection between redox and metabolic circuities is becoming a hot field in cancer biology, as it has the potency to provide selective targets for innovative chemotherapeutic tools that interfere with metabolic and/or redox adaptations of cancer cells. In this Research Topic we have assembled a collection of review articles that, we hope, will help the readers obtain a broad overview on different aspects of cancer metabolism and redox signaling. Moreover, we have included a substantial number of original research papers offering new insights on redox/metabolic pathways of cancer cells.

OPEN ACCESS

Edited and reviewed by:
Paolo Pinton,
University of Ferrara, Italy

***Correspondence:**
Giuseppe Filomeni
filomeni@bio.uniroma2.it

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *09 August 2018*
Accepted: *04 September 2018*
Published: *26 September 2018*

Citation:
Rizza S, Rasola A, Townsend DM and Filomeni G (2018) Editorial: Redox and Metabolic Circuits in Cancer. Front. Oncol. 8:403. doi: 10.3389/fonc.2018.00403

Zulato et al. provide evidence that down-regulation of the liver kinase B1 (LKB1) impacts on cancer cell redox signaling by perturbing the expression of several genes involved in ROS homeostasis. In particular, they found out that LKB1 loss induces NADPH oxidase 1 (NOX1) transcription, thus effecting cell redox state and sustaining tumorigenicity of LKB1-deficient tumors. Indeed, NOX1 inhibition is able to counteract ROS formation, angiogenesis and growth of LKB1-deficient tumor xenografts in mice. Another mechanism by which cancer cells adapt to changes in their redox homeostasis is described in the brief report by Piras et al. They demonstrate how, in aggressive undifferentiated neuroblastoma, the miRNA-494 is involved in the regulation of heme oxygenase 1 (HO-1), a crucial enzyme affecting cell adaptation to oxidative stress and playing an important role in cancer progression and resistance to therapies. In addition, Koundouros and Poulogiannis comprehensively report on the involvement of ROS metabolism and metabolic rewiring in tumorigenesis driven by phosphoinositide the 3-kinase (PI3K)/AKT axis, one of the most frequently deregulated signaling pathways in cancer. The Authors elaborate on different aspects, ranging from the activation of NADPH oxidases (NOXs) to the redox-dependent inactivation of the phosphatase and tensin homolog (PTEN); from the mechanisms through which PI3K/Akt activation helps maintaining redox adaptation of cancer, to the opportunities for therapeutically exploiting redox metabolism in hyperactive PI3K/Akt tumors. The interplay between metabolism rewiring of tumor cells and oncogenic driver mutations is further discussed by De Santis et al., who analyze the crosstalk among mutations in oncogenes (i.e., PI3K/AKT/mTOR, RAS pathway and MYC), tumor suppressors (i.e., p53 and LKB1), cancer cell metabolism and response to therapy.

From a different viewpoint, Stagni et al. focus on the emerging role of a renowned player in the DNA damage response, Ataxia Telangiectasia Kinase (ATM), in redox cancer biology. In this review article, the Authors highlight the complexity of the molecular circuits through which ATM modulates cancer progression by interfering with redox homeostasis and mitophagy in a DNA damage-independent way. ATM mutations, alongside the effects they produce on genome stability, affect mitochondria homeostasis and trigger ROS formation. On the other hand, ATM hyper-activation sustains survival of cancer stem cells by promoting autophagy. Regarding this last process, the role of autophagy in cancer is still debated. It can, indeed, act as a tumor-suppressor during the early stages of tumorigenesis whereas, in established tumors, it sustains the removal of damaged organelles, thus helping cell proliferation, and facilitating drug resistance. The review article from Ichimura and Komatsu discusses on the role of autophagy as major cellular defense mechanism against metabolic and oxidative stress in relation with the Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor (erythroid-derived 2)-like 2 (Nrf2) system, the master regulator of the antioxidant transcriptional response. Autophagy and the Keap1/Nrf2 system are interconnected *via* the phosphorylation of the autophagy receptor protein p62/SQSTM1. The Authors provide an overview on recent findings indicating that p62-Keap1-Nrf2 axis drives cell growth and drug resistance in premalignant cells by promoting metabolic reprogramming.

Besides the established implication of ROS in neoplastic transformation and progression, in the last decades a prominent role for nitric oxide (NO) and *S*-nitrosylation in carcinogenesis has emerged. In this context, Rizza and Filomeni offer a new perspective on the role of denitrosylases, mainly S-nitrosoglutathione reductase (GSNOR), in human cancer, whereas Papaleo's group provides an extensive review article Bignon et al. focusing on the mechanisms of *S*-nitrosylation from a structural and computational point of view, pointing to the main cancer-related targets of *S*-nitrosylation so far identified.

Since the pioneering studies of Otto Warburg, it is clear that most cancer cells preferentially use glycolysis to sustain their high rate of ATP production, even in the presence of normal oxygen tension. Such a metabolic rewiring is commonly referred to as aerobic fermentation, or "Warburg effect," and it is regulated by several transcription factors, among which the hypoxia-inducible factor 1α (HIF-1α) is one of the most relevant. Dysregulations of HIF-1α expression have been, indeed, implicated in processes such as angiogenesis, energy metabolism, cell survival, and tumor invasion. The oncogenic role of HIF-1α derives from his position at the crossroad between glucose metabolism, oxygen availability, redox stress, and gene transcription regulation. In this research topic, Laitala and Erler elaborate on a new role of HIFs in regulating the extracellular matrix. Cancer progression is, actually, controlled by tumor microenvironment, and hypoxic conditions seem to affect the tumor niche where stromal and cancer cells are in close contact. The original research article by Hlouschek et al. focuses on the resistance of lung cancer cells to radio- and chemotherapy due to chronic cycling hypoxia/reoxygenation stress. They show that this condition is able to trigger the up-regulation of the mitochondrial citrate carrier SLC25A1, impacting on glutathione levels and inducing radio-resistance. Coherently, they provide data suggesting that SLC25A1 inhibitors might sensitize tumor to radiotherapy.

As master regulators of metabolic fluxes, mitochondria play a crucial role in tumor biology. Cannino et al. performed an extensive analysis of the crosstalk of metabolic signals between mitochondria and rest of the cell, focusing on how mitochondrial bioenergetic circuitries can tune the metabolic requirements of cancer cells to the fluctuating environmental conditions. In the short review from Esparza-Moltó and Cuezva, the readers will find an overview on the role of H^+-ATP synthase and its physiological inhibitor, the ATPase Inhibitory Factor 1 (IF1), whose over-activation in several human cancers is associated with energy metabolism reprogramming and mitochondrial ROS production. Mitochondrial homeostasis has been also investigated by Gibellini et al., who focus on the role of modulation of the mitochondrial Lon protease (LonP) in metastatic colon cancers. They show that LonP1 is poorly expressed in normal mucosa, while it increases gradually from aberrant crypt foci to adenoma, becoming highly abundant in established colorectal cancers. LonP1 expression seems to correlate with mitochondrial dysfunctions, the rate of glycolysis and pentose phosphate pathway, this seemingly enhancing the epithelial-mesenchymal transition.

In this Research Topic, we have also dealt with some therapeutic aspects of cancer cell redox balance and metabolism. Along this line, the original research by Mattarei et al. explores the utility of novel mitochondria-targeted furocoumarin derivatives as possible anti-cancer agents. The Authors synthesized and tested the efficacy of a neo-synthesized coumarin derivative that blocks the potassium channel Kv1.3, this inducing oxidative stress and cytotoxicity in several malignant cells. Lettieri-Barbato and Aquilano elaborate on the effects that diet can have on cancer cells sensitization to conventional cancer therapies, while simultaneously protecting normal cells from their side effects. The Authors review the recent advances in cancer therapy focusing on the effects of adjuvant dietary interventions, and theorize a novel nutritional approach based on moderate ketogenic diets that could be exploited for future pre-clinical research in cancer therapy.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

Danish Cancer Society Grant Kræftens Bekæmpelse Videnskabelige Udvalg (KBVU) (grant R146-A9414 to GF); Associazione Italiana per la Ricerca sul Cancro (AIRC grant IG2018/20719 to GF and IG 2017/20749 to AR); University of Padua (to AR); Children's Tumor Foundation Drug Discovery Initiative (grant 2016A-05-009, to AR); Neurofibromatosis Therapeutic Acceleration Program (to AR); Piano for Life Onlus and Linfa Onlus (to AR).

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 24 August 2018
doi: 10.3389/fonc.2018.00333

Metabolic Plasticity of Tumor Cell Mitochondria

*Giuseppe Cannino†, Francesco Ciscato†, Ionica Masgras, Carlos Sánchez-Martín and Andrea Rasola**

Department of Biomedical Sciences, University of Padova, Padova, Italy

Mitochondria are dynamic organelles that exchange a multiplicity of signals with other cell compartments, in order to finely adjust key biological routines to the fluctuating metabolic needs of the cell. During neoplastic transformation, cells must provide an adequate supply of the anabolic building blocks required to meet a relentless proliferation pressure. This can occur in conditions of inconstant blood perfusion leading to variations in oxygen and nutrient levels. Mitochondria afford the bioenergetic plasticity that allows tumor cells to adapt and thrive in this ever changing and often unfavorable environment. Here we analyse how mitochondria orchestrate the profound metabolic rewiring required for neoplastic growth.

Keywords: mitochondria, tumor metabolism, signal transduction, oxidative phosphorylation, neoplastic growth, oncometabolites, redox homeostasis, calcium

OPEN ACCESS

Edited by:
Saverio Marchi,
University of Ferrara, Italy

Reviewed by:
Cesar Cardenas,
Universidad de Chile, Chile
Massimo Bonora,
Department of Cell Biology, Albert Einstein College of Medicine, United States

***Correspondence:**
Andrea Rasola
andrea.rasola@unipd.it;
rasola@bio.unipd.it

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *01 June 2018*
Accepted: *02 August 2018*
Published: *24 August 2018*

Citation:
Cannino G, Ciscato F, Masgras I, Sánchez-Martín C and Rasola A (2018) Metabolic Plasticity of Tumor Cell Mitochondria. Front. Oncol. 8:333. doi: 10.3389/fonc.2018.00333

INTRODUCTION

Mitochondria are metabolic hubs that harbor enzymes responsible for several biochemical circuitries, including tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OXPHOS), fatty acid oxidation (FAO), biosynthesis of amino acids, lipids and nucleotides and maintenance of homeostatic levels of Ca^{2+} and of reducing equivalent carriers. These bioenergetic, biosynthetic and signaling functions render mitochondria capable of rapidly sensing and integrating stress signals, in order to coordinate biochemical pathways required for the appropriate responses of the cell to environmental changes (1).

Mitochondria gained center stage in molecular oncology when Otto Warburg observed that tumor cells can ferment glucose to lactate even in the presence of oxygen, proposing that a failure in mitochondrial respiration was the cause of this metabolic trait, called aerobic glycolysis, and that this was in turn required for neoplastic growth (2, 3). Decades after this groundbreaking observation, we know that aerobic glycolysis is part of a wider metabolic rewiring that characterizes neoplastic growth. During this process, environmental conditions can rapidly fluctuate, following local changes in oxygen, pH or nutrient gradients, and can become extremely harsh for the transformed cell, which must become capable of tackling sudden shortages in blood supply or exposure to anti-neoplastic treatments.

Unlike Warburg's proposal, tumor cell mitochondria not only retain their functionality, but are also instrumental for integrating a variety of signals and adjusting the metabolic activity of the cell to such a demanding and stressful situation (4) (**Figure 1**). OXPHOS activity is down-regulated, but not abolished, in many tumor cell types. Therefore, malignant cells start producing a large portion of their ATP through glycolysis rather than OXPHOS. Enhanced glucose utilization also increases the metabolic flux through pentose phosphate pathway (PPP) (5), which provides anabolic building blocks for nucleotide synthesis and NADPH for anti-oxidant defenses, whereas glycolytic intermediates are used for the biosynthesis of amino acids (4, 6–8). These metabolic

changes down-regulate the TCA cycle, both because induction of PPP and of anabolic pathways that branch from glycolysis limit pyruvate availability, and because a low OXPHOS activity inhibits the formation of NAD^+ and FAD required for TCA cycle dehydrogenases. Thus, mitochondria must activate anaplerotic mechanisms in order to feed the TCA cycle, as its activity is required for fatty acid (FA) and amino acid biosynthesis and for the homeostatic maintenance of reducing equivalent carriers (6, 9–14); this is mainly achieved by increasing the pace of glutamine utilization (9, 15) (**Figure 2**).

Several recent lines of evidence suggest that mitochondria indeed play a key promoter role in tumor growth and progression (16). All along this process, mitochondrial biogenesis and quality control are often upregulated, and mitochondria can even retain a high level of OXPHOS in some tumor cell types. Rare human neoplasms with defective respiration caused by mutations in mitochondrial genome, such as oncocytomas (17, 18), are relatively benign, and mitochondrial DNA depletion impairs tumorigenicity in several tumor cell models (19). Altogether, these observations imply the existence of a negative selection for a loss of mitochondrial function in neoplastic transformation (20).

Mitochondrial bioenergetics is largely under the control of extra-mitochondrial biochemical pathways, whose activity is often altered by oncogenic mutations (21). Moreover, some metabolic alterations that directly originate from mitochondria are oncogenic *per se* (22). In certain tumor settings, mitochondria can act as neoplastic drivers by generating high levels of oncometabolites, *i.e.,* metabolites that are able to change the genomic and epigenomic landscape of the cell, hence prompting the tumorigenic process (23, 24). Thus, the crosstalk between mitochondria and rest of the cell can amplify the metabolic drift of tumor cells away from their non-transformed counterparts during neoplastic progression.

In the present review, we analyse how the metabolic plasticity of tumor cell mitochondria contributes to the neoplastic process. However, any general consideration must be confronted with the real scenario of a tumor mass, where a myriad of factors influence metabolism. These include the tissue of origin of the neoplastic cell, its mutational and epigenomic profile and the local environmental conditions, which can dictate confined changes in the bioenergetic features of the cells, prompting metabolic heterogeneity even in different portions of the same tumor, or in different moments of its growth (25).

ONCOGENIC SIGNALLING PATHWAYS AND MITOCHONDRIA

A complex network of signals moves back and forth between nucleus and mitochondria (**Figure 3**). This crosstalk constantly keeps under strict nuclear control any mitochondrial function, ensuring its proper harmonization with the metabolic status of the cell. Several major transduction pathways have a strong impact on mitochondrial function, including the transcriptional programs coordinated by HIF1, c-Myc and p53, as well as Ras and mTOR/AMPK signaling (4, 21, 26, 27). Consequently, pro-neoplastic dysregulation of any of these signaling axes strongly affects the mitochondrial metabolic machinery.

Hypoxia-Inducible Factors (HIFs) and Mitochondrial Metabolism

HIFs induce transcription under low oxygen conditions and are active when their two subunits, aryl hydrocarbon receptor nuclear translocator (ARNT, or HIF-1β) and either HIF-1α or HIF-2α, bind hypoxia-responsive elements (HREs) in gene promoters. While ARNT is constitutively expressed, HIF-1α/2α undergo proteasomal degradation triggered by hydroxylation of specific proline residues. The prolyl-hydroxylases (PHDs) targeting HIF-1α/2α are dioxygenases inhibited in hypoxic or anoxic conditions, which leads to stabilization of HIF-1α and/or HIF-2α. HIF stabilization orchestrates a transcriptional program that equips tumor cells to sustain hypoxic stress by affecting several aspects of cancer biology, including angiogenesis, epithelial-to-mesenchymal transition, metastasis, resistance to anticancer therapies as well as metabolic reprogramming (28–30).

HIF-dependent metabolic rewiring embraces induction of glycolysis and FA synthesis together with OXPHOS down-regulation, a key adaptation to low oxygen (31), and has profound effects on mitochondrial activity (**Figure 3**). One of the glycolytic enzymes induced by hypoxia is hexokinase type II (HK II), the most active hexokinase isoform whose expression is upregulated in many cancer types and contributes to their efficiency in glucose utilization (32, 33). In tumor cells, HK II is mainly anchored to the outer mitochondrial membrane, and its detachment from mitochondria rapidly induces cell death (34–36). Thus, mitochondrial binding of HK II has an important tumorigenic function (37) and displays a protective role for mitochondrial function and cell viability through mechanisms yet poorly defined, but involving autophagy regulation in conditions of glucose paucity (38).

The transcriptional program mastered by HIFs creates a bottleneck in funneling glycolysis toward the TCA cycle by slowing-down the conversion of pyruvate to acetyl-CoA (31). This is achieved both through induction of the M2 isoform of pyruvate kinase (PKM2), which is less active than the M1 counterpart in generating pyruvate from phosphoenolpyruvate, and by eliciting the expression of pyruvate dehydrogenase kinase 1 (PDK1), an inhibitor of the pyruvate dehydrogenase complex (PDC) (39, 40). In addition, HIFs promote lactate dehydrogenase (LDHA) expression, again pushing pyruvate away from the TCA cycle toward its conversion into lactate, using reducing equivalents provided by glycolysis-derived NADH and thus keeping the NAD^+ levels required for a sustained glycolytic activity (41). The parallel induction of monocarboxylic acid transporters (MCTs) causes lactate extrusion from the cell and contributes to acidification of the surrounding environment. As a combined result of these modulations, OXPHOS activity is down-modulated, and glycolytic intermediates upstream to pyruvate accumulate and can be diverted to anabolic routines (42).

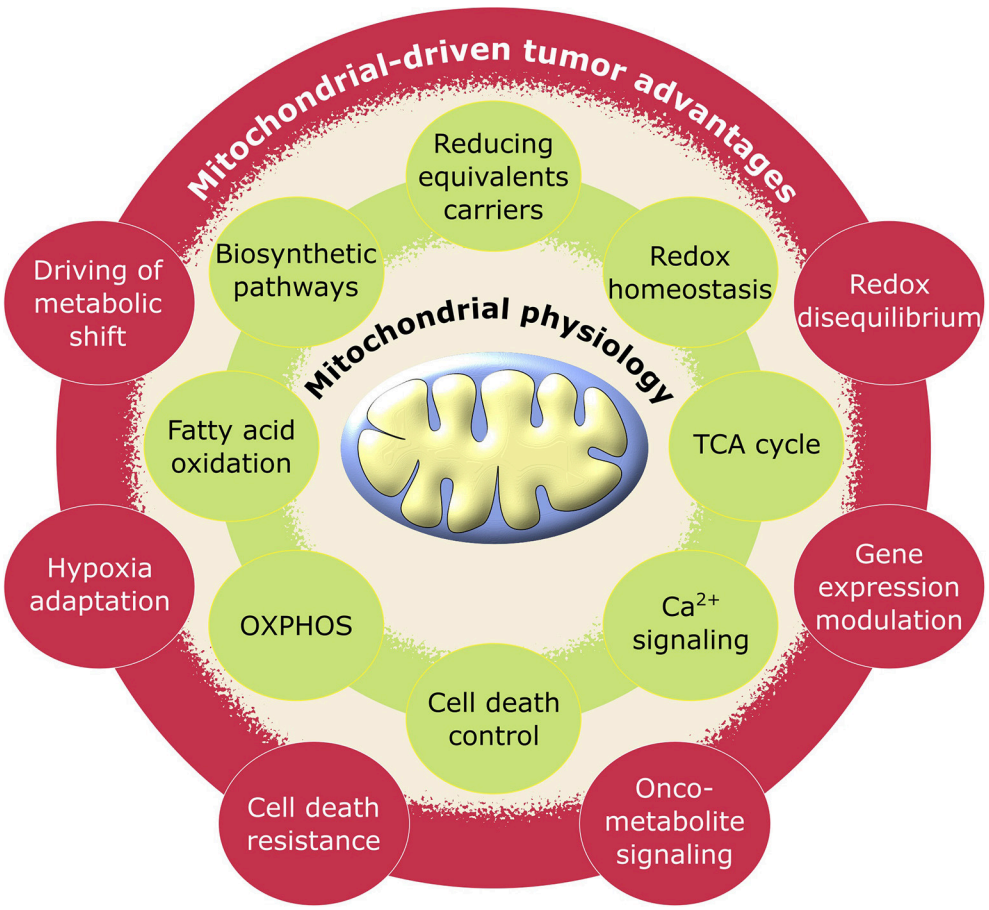


FIGURE 1 | Schematic representation of pro-tumoral biological processes regulated by mitochondria. Mitochondrial physiology (green) acquires advantageous alterations in cancer (red) adjusting its metabolic activity to support the requirements for neoplastic cell growth and proliferation.

In these conditions, tumor cells must use lipids and amino acids as main metabolic fuels (43), finding glucose-independent sources for acetyl-CoA generation required for *de novo* FA synthesis and for acetylation reactions (see section Post Translational Regulation In Cancer Metabolism). In general, tumor cells increase FA synthesis and the intracellular levels of total FAs for membrane synthesis, lipid signaling or as energy source (when oxidized) (44, 45). HIF signaling increases lipid uptake and the induction of lipid kinases and oxidases, resulting in an overall dysregulation of lipid metabolism in cancer (46). To obtain high levels of acetyl-CoA, mitochondria of cells undergoing hypoxia boost reductive carboxylation of glutamine (47), which generates citrate via the TCA cycle enzymes isocitrate dehydrogenase (IDH) and aconitase. Citrate then moves to cytosol, where it can be cleaved into oxaloacetate and acetyl-CoA by ATP citrate lyase (ACLY), thus starting FA synthesis (**Figure 3**). HIF1α causes proteasomal degradation of a subunit of the α-ketoglutarate dehydrogenase (αKGDH) complex, a TCA component that is responsible for oxidative glutamine metabolism, by inducing the E3 ubiquitin-ligase SIAH2 (48). Thus, HIF-dependent transcription enhances reductive carboxylation of glutamine by inhibiting its oxidation. In parallel with induction of FA synthesis, HIF signaling down-modulates FAO both directly, by inhibiting the expression of the mitochondrial enzymes medium- and long-chain acetyl-CoA dehydrogenase (MCAD and LCAD) (49) and indirectly, by inducing PHD3, which activates acetyl-CoA carboxylase 2 (ACC2), thus prompting generation of the FAO repressor malonyl-CoA (50).

Mitochondria can also directly regulate HIF stability in a process termed pseudohypoxia that is independent of environmental oxygen levels and further adds flexibility to the metabolic responses of tumor cells (see section Mutations Of Mitochondrial Enzymes In Cancer Metabolism). Furthermore, at least in a model of renal carcinoma, HIF1α can repress the expression of PGC-1α (peroxisome proliferator-activated receptor gamma, coactivator-1α), a central regulator of mitochondrial biogenesis, which in turn stabilizes HIF1α (51). These observations highlight the existence of regulatory loops between mitochondria and the transcriptional program mastered by HIFs (52). Hypoxia also creates a redox stress in mitochondria, as oxygen is the final electron acceptor in OXPHOS and inadequate oxygen levels increase the leakage of electrons out of respiratory complexes, forming reactive

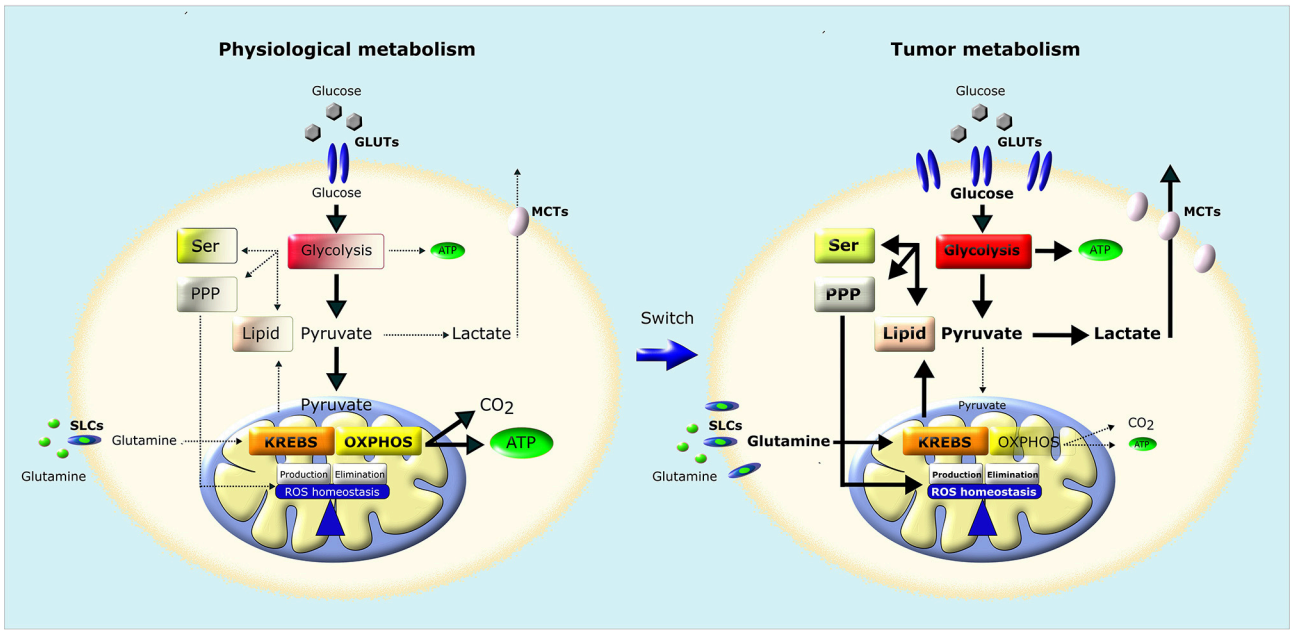


FIGURE 2 | Metabolic remodeling of cancer cells. In normal cells **(left)**, a large fraction of glucose is metabolized to pyruvate that is almost completely oxidized to CO_2 through TCA (Krebs) cycle and OXPHOS in mitochondria, producing a large amount of ATP. Pyruvate is metabolized to lactate only in conditions of limiting O_2. Instead, most cancer cells **(right)** convert most glucose to lactate regardless of O_2 availability (Warburg effect). The increased glucose utilization through glycolysis, associated to an increase in glutamine utilization, generates metabolic intermediates used for the synthesis of nucleic acids through pentose phosphate pathway (PPP), serine biosynthesis pathway (SER) and lipid biosynthesis, providing the building blocks for the anabolic needs of cancer cells. In addition, neoplastic cells undergo an increase in ROS generation, and therefore increase their antioxidant defenses to avoid oxidative damage and maintain ROS homeostasis. GLUTs, Glucose Transporters; MCTs, Lactate Transporters; SLCs, Solute Carriers.

oxygen species (ROS). Therefore, HIF signaling is also involved in the maintenance of redox homeostasis, another complex bioenergetic adaptation required for neoplastic progression in which mitochondrial play a central role (see section Redox Homeostasis And Mitochondrial Metabolism In Tumors).

c-Myc and Mitochondrial Metabolism

c-Myc is one of the most frequently induced oncogenes in human cancers, where its transcriptional function becomes constitutively activated following deregulation of oncogenic pathways, gene amplification or chromosomal translocation (53). The effect of c-Myc activation is the orchestration of nutrient uptake and cell growth and proliferation, making its dysregulation a key oncogenic driver. These biological routines require a robust anabolic induction, and this is crucially supported by mitochondria. There are several ways by which c-Myc affects mitochondrial metabolism, thus sustaining growth of neoplastic cells in the unfavorable environment they must deal with. The transcriptional program mastered by c-Myc partially overlaps the metabolic effects of HIF-dependent signaling. Indeed, c-Myc upregulates the same set of glycolytic genes that are targeted by HIFs, including GLUT1, LDHA, MCTs, PKM2, and HK II, thus increasing glucose uptake and its utilization both in glycolysis and PPP (**Figure 3**). As discussed for HIFs, these changes cause a metabolic rewiring toward aerobic glycolysis, lowering in parallel pyruvate availability for the TCA cycle and OXPHOS (54).

At variance from HIFs, however, c-Myc is active under non-hypoxic conditions, and can stimulate mitochondrial biogenesis and respiration. c-Myc activates mitochondrial transcription factor A (TFAM), PGC1β and mitochondrial DNA polymerase gamma, which elicit the expression of hundreds of genes encoding for mitochondrial proteins (55). This could be relevant for the local adaptations of tumor cells to the microenvironmental heterogeneity they find in the tumor mass. It is possible to envision that c-Myc can prompt both glycolysis and OXPHOS in neoplastic cells located in the proximity of blood vessels, where high levels of oxygen are available. Instead, when cells encounter more hypoxic conditions, c-Myc could cooperate with HIFs in increasing glycolysis and attenuating mitochondrial OXPHOS, without inhibiting other mitochondrial metabolic activities (56).

Induction of mitochondrial serine hydroxymethyltransferase (SHMT2) by c-Myc provides an elegant example of this conditional cooperation between c-Myc and HIFs in regulating metabolic circuitries of tumor cell mitochondria. SHMT2 is the major source of the one-carbon unit required for folate metabolism and for the biosynthesis of nucleotides and amino acids (**Figure 3**). It utilizes serine, obtained from the glycolytic intermediate 3-phosphoglycerate, and tetrahydrofolate (THF) to catalyze the synthesis of glycine and 5,10-methylenetetrahydrofolate (5,10-CH2-THF). In turn, 5,10-CH2-THF can generate formate and the reducing equivalent donor NADPH in a reaction catalyzed

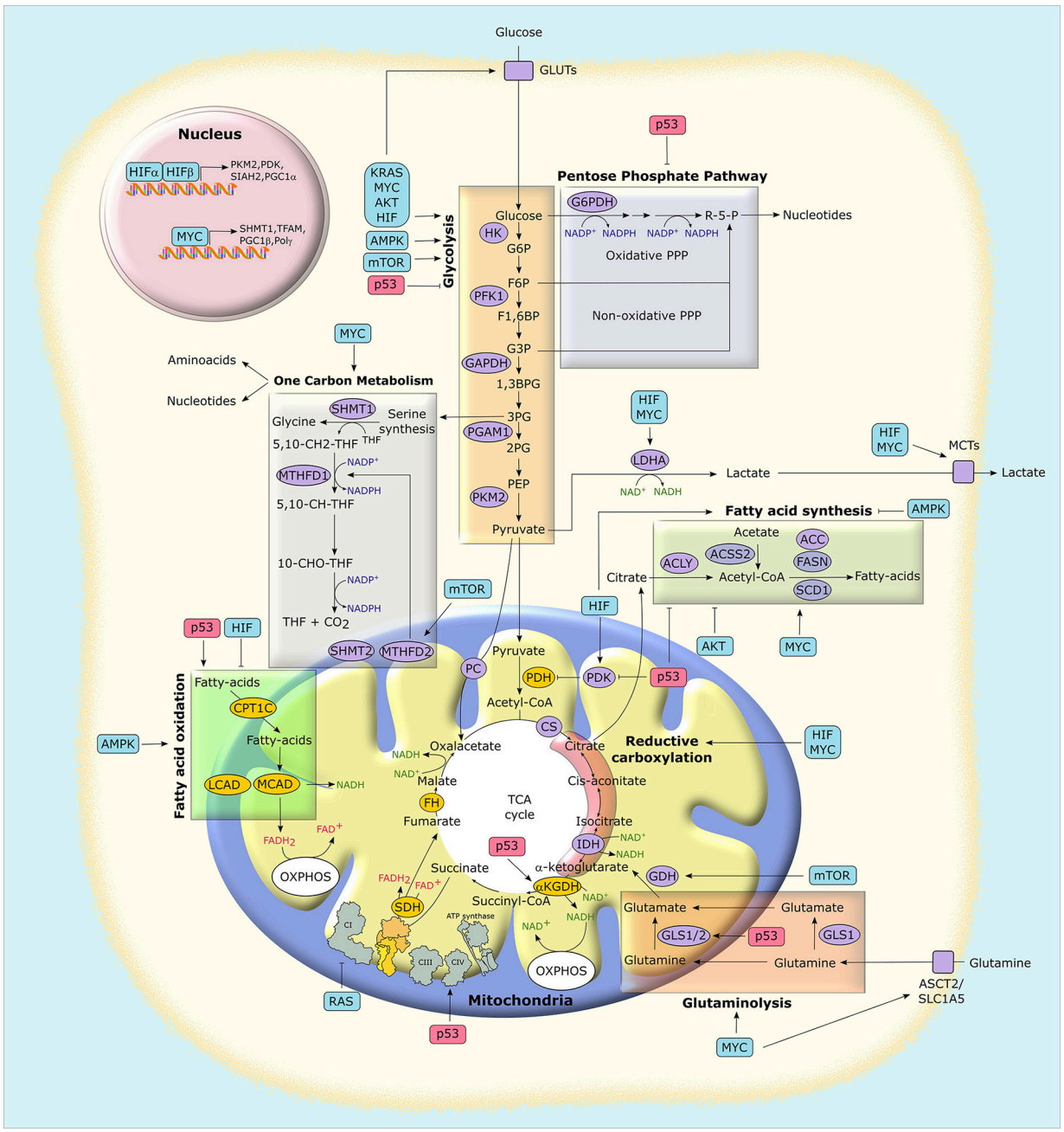


FIGURE 3 | Mitochondria at the crossroad of metabolic networks and signaling cascades. Several proteins with pro-neoplastic activity (in light blue boxes) alter the expression and/or the activity of metabolic enzymes and transporters, thus rewiring the metabolic status of cancer cells. Similarly, the possible loss of the tumor suppressor p53 (in light red boxes) impacts on cancer metabolism at several levels. Enzymes that catalyze metabolic reactions are shown in ovals. Yellow ovals indicate enzymes that are preferentially inhibited in tumors, while purple ovals indicate those that are mostly induced. G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F1,6BP, fructose-1,6-bisphosphate; G3P, glyceraldehyde-3-phosphate; 1,3BPG, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phospho-enolpyruvate; R-5-P, ribose-5-phosphate; MCT, monocarboxylate transporter; GLUT, glucose transporter; PC, pyruvate carrier; ASCT2, alanine, serine, cysteine-preferring transporter 2; 5,10-CH2-THF, 5,10-methylenetetrahydrofolate; 10-CHO-THF, 10-formyl-THF; 5,10-CH-THF, 5,10 methenyl-THF; HIF, hypoxia-inducible factor; HK, hexokinase; PFK1, phosphofructokinase 1; PGAM1, phosphoglycerate mutase 1; PKM2, pyruvate kinase M2 isoform; LDHA, lactate dehydrogenase A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PDH, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; αKGDH, α-ketoglutarate dehydrogenase; CS, citrate synthase; SDH, succinate dehydrogenase; FH, fumarate hydratase; IDH, isocitrate dehydrogenase; GLS, glutaminase; ACLY, ATP-citrate synthase; ACSS2, Acyl-coenzyme A synthetase short-chain family member 2; ACC, acetyl-CoA carboxylase; FASN, fatty acid synthase; SCD1, stearoyl-CoA desaturase 1; SHMT, serine hydroxymethyltransferase; MTHFD, methylenetetrahydrofolate dehydrogenase; CPT, carnitine O-palmitoyltransferase; MCAD, medium-chain acetyl-CoA dehydrogenase; LCAD, long-chain acetyl-CoA dehydrogenase; PGC1, peroxisome proliferator-activated receptor gamma, coactivator-1; TFAM, mitochondrial transcription factor A; PPP, pentose phosphate pathway; TCA, tricarboxylic acid; OXPHOS, oxidative phosphorylation.

by methylenetetrahydrofolate dehydrogenase 2 (MTHFD2). c-Myc-dependent induction of SHMT2 under normoxic conditions has important biosynthetic consequences: formate is released in the cytosol where it is involved in purine synthesis; glycine and 5,10-CH2-THF contribute to nucleotide synthesis, and NADPH is required for reductive biosynthesis of amino acids, deoxyribonucleotides and lipids (54, 55). When a tumor cell faces hypoxia, HIF stabilization further induces SHMT2. This is counterintuitive, as in low-oxygen conditions cells inhibit proliferation, thus reducing demand for anabolic fluxes. Nevertheless, under hypoxia SHMT2 is essential for survival of c-Myc-transformed cells as it protects them from oxidative stress. Indeed, MTHFD2 activity maintains a high $NADPH:NADP^+$ ratio, and NADPH is required for regeneration of the antioxidant tripeptide glutathione and hence for protection from ROS damage (57). Notably, SHMT2 is required for survival and proliferation of neoplastic cells in ischemic tumor zones (58).

c-Myc also promotes the glutamine addiction that characterizes several cancer cell types (54, 55) (**Figure 3**). Glutamine is both a nitrogen and carbon source essential for biomass accumulation, and a substrate used for bioenergetic purposes, and it is avidly consumed by neoplastic cells for proliferation and survival (**Figures 2**, **3**). c-Myc increases the expression of the plasma membrane glutamine transporter, ASCT2/SLC1A5, and of glutaminases (GLS) that convert glutamine to glutamate in cytosol (GLS1) or mitochondria (GLS2) as a first step of its oxidation (15, 59). Glutamate generates α-ketoglutarate (α-KG) either via glutamate dehydrogenase (GDH), in a reaction that releases ammonia, or via several aminotransferases that transfer glutamate nitrogen to α-keto acids, such as pyruvate, for producing other amino acids and α-KG. In turn, α-KG feeds the TCA cycle, which is therefore accelerated by c-Myc via glutaminolysis induction. c-Myc also increases the TCA cycle flux up to four-folds by eliciting the expression of most of its enzymes (55). When the TCA cycle flux is impaired, e.g., in low glucose conditions or by mutations in some of its components, α-KG can act as an anaplerotic substrate that provides carbon units to yield citrate by moving backwards through the TCA cycle through reductive carboxylation (9). In cytosol, citrate starts lipid synthesis. c-Myc induces the string of enzymes responsible for the first steps of lipidogenesis, whose upregulation occurs across most tumors (45). These enzymes include ACLY, which uses citrate to synthesize acetyl-CoA, Acetyl-CoA carboxylase, which generates malonyl-CoA, fatty acid synthase and stearoyl-CoA desaturase (55). Malonyl-CoA inhibits carnitine acyl transferase I (*aka* carnitine palmitoyltransferase I, CPT I), the carrier responsible for the uptake of fatty acids in mitochondria, thus acting in a feedback loop on mitochondrial metabolism to inhibit FAO (60).

Taken together, these observations demonstrate how tumor cell mitochondria can control the homeostatic balance of reducing equivalent donors, OXPHOS activity, lipid synthesis and oxidation in response to c-Myc and/or HIF activation, with crucial implications for chromatin remodeling, tuning of all major anabolic pathways and handling of oxidative insults.

p53 and Mitochondrial Metabolism

The transcription factor p53 is a key tumor suppressor activated by a set of stress signals, such as genotoxic damage, oncogene activation, nutrient or oxygen scarcity and loss of cell-to-cell contacts, all of which characterize malignant transformation (61, 62). A functional inactivation of p53 occurs in the majority of tumor types (63). p53 mainly exerts its activity in the nucleus by regulating the expression of genes or microRNAs (miRNAs), even though it can also act in cytosol and mitochondria to inhibit autophagy (64) or to promote cell death (65). p53 activation induces death or senescence when a sustained or intense stress causes irreversible cell damage. Conversely, mild stresses result in p53-dependent adaptive responses, consistent with its role in repairing or avoiding damage. During fluctuations in oxygen or nutrient availability, the effect of p53 is a global promotion of cell catabolism, associated with an inhibition of proliferation and growth (**Figure 3**). p53 interacts with mTOR (mammalian target of rapamycin) and AMPK (AMP-activated protein kinase), two master regulators of cellular metabolism. The mTORC1 complex is active in the presence of both adequate growth conditions and mitogens and coordinates the anabolic responses of the cell (66), whereas AMPK inhibits mTORC1 and allows cells to adapt to energetic stresses by utilizing glucose and FA for increasing ATP levels (see below) (67). A complex interplay exists between p53 and the mTORC1/AMPK circuitry: p53 both activates AMPK and elicits the expression of several negative regulators of mTORC1, whereas AMPK activates p53 through several means, including its phosphorylation and acetylation (62, 68). In this way, cells can respond in a balanced and flexible way to metabolic variations, different types of stress signals, growth and proliferation inputs (69).

p53 can also exert a more direct action on several metabolic effectors (**Figure 3**). In general, it dampens the glycolytic rate through a concerted downregulation of glucose transporters (GLUTs) (70) and of the glycolytic enzyme phosphoglycerate mutase (PGAM) (71). p53 also prompts the expression of TIGAR (TP53-induced glycolysis and apoptosis regulator), which indirectly inhibits phosphofructose kinase 1 (PFK1) (72), thus leading to a p53-dependent diversion of glycolytic intermediates into the PPP (62). However, the effect of p53 on glucose metabolism is highly context-dependent, as p53 can also negatively modulate PPP activity (73). In parallel to inhibiting glycolysis, p53 enhances mitochondrial bioenergetic activity in several ways (**Figure 3**). It promotes mitochondrial quality control by inducing mitophagy to substitute damaged mitochondria, and it increases mitochondrial DNA copy number and mitochondrial mass (68). p53 boosts TCA cycle via induction of the mitochondrial glutaminase GLS2 (74, 75), thus fueling glutamine to glutamate conversion and in turn α-KG generation through the TCA cycle enzyme α-KGDH. Moreover, p53 activates the PDC as it inhibits the negative PDC regulator pyruvate dehydrogenase kinase 2 (76), increasing pyruvate funneling into the TCA cycle through its conversion to acetyl-CoA. p53 also enhances OXPHOS activity by inducing the expression of subunit I of cytochrome c oxidase (COX), the complex IV of the electron transport chain, and of

the COX assembly factor SCO2, as well as of apoptosis-inducing factor (AIF), which is required for a proper OXPHOS functioning (68).

In general, p53 lifts lipid utilization inhibiting FA synthesis and increasing mitochondrial FAO in a coordinated manner (68). This results from p53-dependent modulation of glycolysis and PPP, as these routines supply the building blocks (acetyl-CoA and NADPH) needed for lipid synthesis. Furthermore, p53 directly stimulates FAO by inducing the mitochondrial membrane FA transporters carnitine acetyltransferases (CPTs) (77, 78), the transcriptional co-activator for FAO genes lipin 1 (79), and pantothenate kinase 1, which is essential in β-oxidation as it is involved in CoA biosynthesis (80). Moreover, p53 induces malonyl-CoA decarboxylase, which lowers the levels of the CPT allosteric inhibitor malonyl-CoA (81). Thus, p53 opposes the metabolic shift toward the induction of FA synthesis and glycolysis that characterizes many tumor cell types and supports mitochondrial FAO and OXPHOS. Notably, FAO induction further feeds OXPHOS via generation of FADH2 and NADH. Nonetheless, under conditions of extreme stress p53 can have an opposite effect on mitochondria, potently contributing to their dysfunction through repression of the master regulators of mitochondrial biogenesis PGC-1α/PGC-1β, thus leading to cell senescence or death (82).

The AMPK/mTOR System and Mitochondrial Metabolism

AMPK acts as a homeostatic device whose purpose is the rapid restoration of energy balance through an orchestrated inhibition of ATP-consuming biosynthetic pathways. It activates following a drop in ATP levels, as it senses the AMP/ATP ratio. Therefore, AMPK induction is promoted by down-regulation of mitochondrial OXPHOS activity; in turn, AMPK induces glucose uptake and glycolysis while inhibiting storage of glucose, and it suppresses FA synthesis by phosphorylating and inhibiting ACCs (83) (**Figure 3**). ACC2, the isozyme associated to the outer mitochondrial membrane, generates malonyl-CoA that inhibits CPT1 and therefore mitochondrial import of FAs for their oxidation. Hence, ACC2 inhibition by AMPK induces mitochondrial FAO (67). Moreover, AMPK inhibits *de novo* lipid generation via direct phosphorylation of SREBP1 (sterol regulatory element binding protein 1), a master transcriptional regulator of lipid synthesis (84). AMPK also prompts mitophagy through phosphorylation of ULK kinases (1) and mitochondrial fragmentation through phosphorylation of mitochondrial fission factors (85) in order to restrict energy expenditure and to maintain cell viability in conditions of starvation or of OXPHOS dysfunction. When the bioenergetic stress lingers on, AMPK reprograms metabolism for allowing cells to tackle prolonged energy crises. Therefore, a sustained AMPK activation increases FAO as a bioenergetic source, while limiting glucose and lipid synthesis (67) and increasing mitochondrial biogenesis via PGC-1α, thus allowing a high degree of metabolic plasticity to cells undergoing energetic stress (1).

The protein complex mTORC1 is formed by the serine/threonine protein kinase mTOR, by the regulatory proteins Raptor, which facilitates substrate recruitment to mTORC1, and mLST8, which associates with the catalytic domain of mTORC1, and by the two inhibitory subunits PRAS40 and DEPTOR (86). mTORC1 promotes all major anabolic pathways, including protein, lipid and nucleotide synthesis, together with glucose metabolism and organelle biogenesis. It is activated by the Ras/ERK and by the PI3K/Akt signaling pathways, which are deregulated in most cancer types, whereas it is inhibited by the tumor suppressors p53 and LKB1. Thus, mTORC1 induction is a crucial event in the metabolic rewiring of tumor cells (66) (**Figure 3**). mTORC1 promotes a shift from OXPHOS to glycolysis, and it enhances the expression of many glycolytic genes by increasing HIF1α translation (87); in addition, it activates GDH, thus inducing glutaminolysis (88), and it upregulates MTHFD2 expression and therefore the mitochondrial folate pathway required for purine synthesis (89). The mTORC1 complex also activates SREBP, thus opposing the effect of AMPK on lipid metabolism and promoting lipidogenesis (90).

Ras and Mitochondrial Metabolism

RAS genes encode a family of GTPases whose mutations are frequently causative of tumor development and are common in cancer. Hyperactivation of Ras-induced transduction pathways boost cell growth and proliferation, prompt resistance to death signals and promote the acquisition of invasive and metastatic properties (91, 92). Moreover, deregulated Ras signaling orchestrates pro-neoplastic changes in several components of the tumor microenvironment, including cancer-associated fibroblasts, endothelial, inflammatory and immune cells (93). Therefore, Ras mutations are among the most problematic oncogenic events, frequently associated with a dismal prognosis (94). Oncogenic activation of Ras signaling has a profound impact on the metabolic changes of tumor cells (**Figure 3**). It induces HIF1α both by upregulating the HIF1A gene and by enhancing translation of its mRNA via activation of the mTORC1 complex through the Ras downstream effectors ERK and PI3K/Akt (91). As described above, HIF1α in turn promotes both the transport and the glycolytic utilization of glucose and the funneling of glycolytic intermediates into the PPP (95).

In parallel, oncogenic Ras signaling affects mitochondrial bioenergetics in several ways. It prompts mitochondrial translocation of phosphoglycerate kinase I, which inhibits PDC via activation of the PDC inhibitor PDK1; as a result, less pyruvate is funneled in the TCA cycle, leading to a decrease in ROS levels together with a surge in lactate extrusion (96). K-Ras-dependent transformation inhibits OXPHOS by down-regulating respiratory complex I content (97, 98). ERK, a crucial Ras effector that can locate in mitochondria (35, 99), decreases the activity of respiratory complex II, *aka* succinate dehydrogenase (SDH; see also section Post Translational Regulation In Cancer Metabolism) (100). Nonetheless, transformation by Ras does not abrogate OXPHOS activity, which still generates a large fraction of cellular ATP (101–103).

Several evidences underline the importance of mitochondrial activity in Ras-driven tumor cells. Together with loss of p53, hyperactive Ras elicits autophagy in a non-small-cell lung cancer setting, thus preserving a proper mitochondrial function that is required for lipid homeostasis and tumor growth (104).

Inhibition of the mitochondrial transcription factor Tfam leads to mitochondrial depletion and impedes growth of K-Ras-dependent lung tumors (105). Similarly to Myc-transformed cells, Ras-driven cancers strongly rely on glutamine for growth and survival (106, 107), and glutamine is the major carbon source for the TCA cycle when Ras is activated (95). In pancreatic cancer, which is characterized by activating mutations in K-Ras in more than 90% of cases, mitochondrial glutamate-oxaloacetate transaminase 2 (GOT2) utilizes glutamine-derived glutamate and oxaloacetate (OAA) to generate aspartate that is then exported to cytosol. Here, GOT isoform 1 converts back aspartate to OAA, which provides malate and finally pyruvate by malic enzyme (ME). This reaction produces NADPH, a key factor to reduce glutathione in order to avoid oxidative stress (108). Anchorage-independent growth of K-Ras-transformed cells requires α-KG generation by glutamine through glutaminase and alanine aminotransferase activity (105). Lung cancer cells exhibit a high channeling of glycolytic metabolites into the TCA cycle and glutathione biosynthesis, leading to protection from oxidative insults. This metabolic rewiring occurs when neoplastic cells are homozygous for K-Ras^{G12D}, but not in heterozygous K-Ras^{G12D} cells, which characterize early tumor stages, thus highlighting the importance of oncogenic Ras in shaping metabolic heterogeneity and adaptions of cancer cells (109).

MUTATIONS OF MITOCHONDRIAL ENZYMES IN CANCER METABOLISM

The oncogenic role played by changes in mitochondrial metabolism was first spotted by finding that mutations in subunits of SDH, an enzyme placed at the crossroad between OXPHOS and TCA cycle, as well as in the TCA cycle enzyme fumarate hydratase (FH), are causative of some human tumor types (110–113). These were paradigm-shifting findings, as they demonstrated for the first time that mitochondria, and in particular core bioenergetic enzymes, could play an active role in the complex alterations of biochemical circuitries that lead to tumor onset (114). SDH and FH act as classical tumor suppressors (**Figure 4**). Inactivating mutations in SDH subunits have been identified in both genetic and sporadic cancer types, including familial paraganglioma/pheochromocytoma (PGL/PCC) (115), renal carcinoma, thyroid cancer, neuroblastoma, gastrointestinal stromal tumor, ovarian cancer and testicular seminoma (116), whereas FH loss-of-function hallmarks hereditary leiomyomatosis and renal cell cancer (HLRCC) and skin and uterine leiomyomas (22).

Research in the last few years allowed understanding that the metabolic origin of this peculiar subset of neoplasms illustrates concepts of general importance. Indeed, it emerged that there is a tight intertwining between metabolism and gene expression, as cells and tissues (and neoplasms make no exception) must strictly coordinate genome expression and metabolic state for the proper unfolding of their biological routines (117). Components of intermediary metabolism affect the activity of chromatin-modifying enzyme (**Figure 4**). This leads to epigenomic changes mastering pro-neoplastic molecular rewiring via gene expression regulation (118). Inactivating mutations of SDH and FH drive such mechanisms by causing accumulation of their substrates succinate and fumarate, respectively, which leads to inhibition of a class of enzymes called α-KG–dependent dioxygenases. To hydroxylate their substrates, these enzymes take up one oxygen atom by α-KG, which therefore acts as a co-substrate that is decarboxylated and releases carbon dioxide and succinate. Both succinate and fumarate competitively inhibit α-KG–dependent dioxygenases, including the JmjC domain-containing demethylases (KDMs), which hydroxylate lysine residue on histones, the TET (ten-eleven translocation) family of 5-methylcytosine hydroxylases, which induce DNA demethylation of CpG islands near gene promoters, and prolyl hydroxylases (PHDs), which prompt proteasomal degradation of HIF1α (119). These enzymes play central roles in epigenetic and transcriptional regulation, and their inhibition by high levels of succinate and fumarate suppresses differentiation and promotes proliferation and further metabolic changes (20), thus inducing tumorigenesis. As a consequences of these pro-neoplastic effects, both succinate and fumarate have been dubbed oncometabolites (23, 24) and similar patterns of epigenomic changes can be observed both in FH- and in SDH-deficient tumors (120). Oncometabolites also favor tumor cell motility by affecting extracellular matrix composition via inhibition of collagen prolyl-4-hydroxylases (119) and elicit angiogenesis through succinate-dependent transcriptional induction of VEGF (116).

Fumarate inactivates aconitase 2 (**Figure 4**), a TCA cycle enzyme containing an iron-sulfur group (121) and proteins involved in the biogenesis of iron-sulfur clusters (122), and it inhibits the enzymatic activity of SDH through a product inhibition effect, leading to down-modulation of both OXPHOS and TCA cycle function. In order to cope with the absence of a functional TCA cycle, FH-deficient cells utilize fumarate in a linear pathway starting with glutamine and ending with the biosynthesis and degradation of haem and eventually with bilirubin excretion. This pathway is crucial for mitochondrial NADH production, and makes FH-deficient cells dependent on heme oxygenase activity, thus creating a metabolic vulnerability (123). In addition, TET inhibition by fumarate causes inactivating hypermethylation of the anti-metastatic miRNA cluster miR-200, thus promoting the transcriptional program mastered by the miR-200 target ZEB1. ZEB1 induces epithelial-to-mesenchymal transition (EMT), a complex biological rearrangement by which tumor cells acquire invasive properties (124). Thus, fumarate-dependent inhibition of miR-200 boosts EMT (125) (**Figure 4**). Fumarate can also tune cell redox equilibrium via protein succination, *i.e.,* the interaction between fumarate and cysteine residues to produce S-(2-succino)-cysteine, which hampers protein function (24). Fumarate both inactivates by succination Kelch-like ECH-associated protein 1 (KEAP1), which promotes proteasomal degradation of NRF2, the master transcriptional regulator of the cell response to oxidative stress (23, 126), and generates succinated glutathione, an alternative substrate to glutathione reductase that leads to an increase in mitochondrial ROS levels and to HIF-1 activation (127) (**Figure 4**).

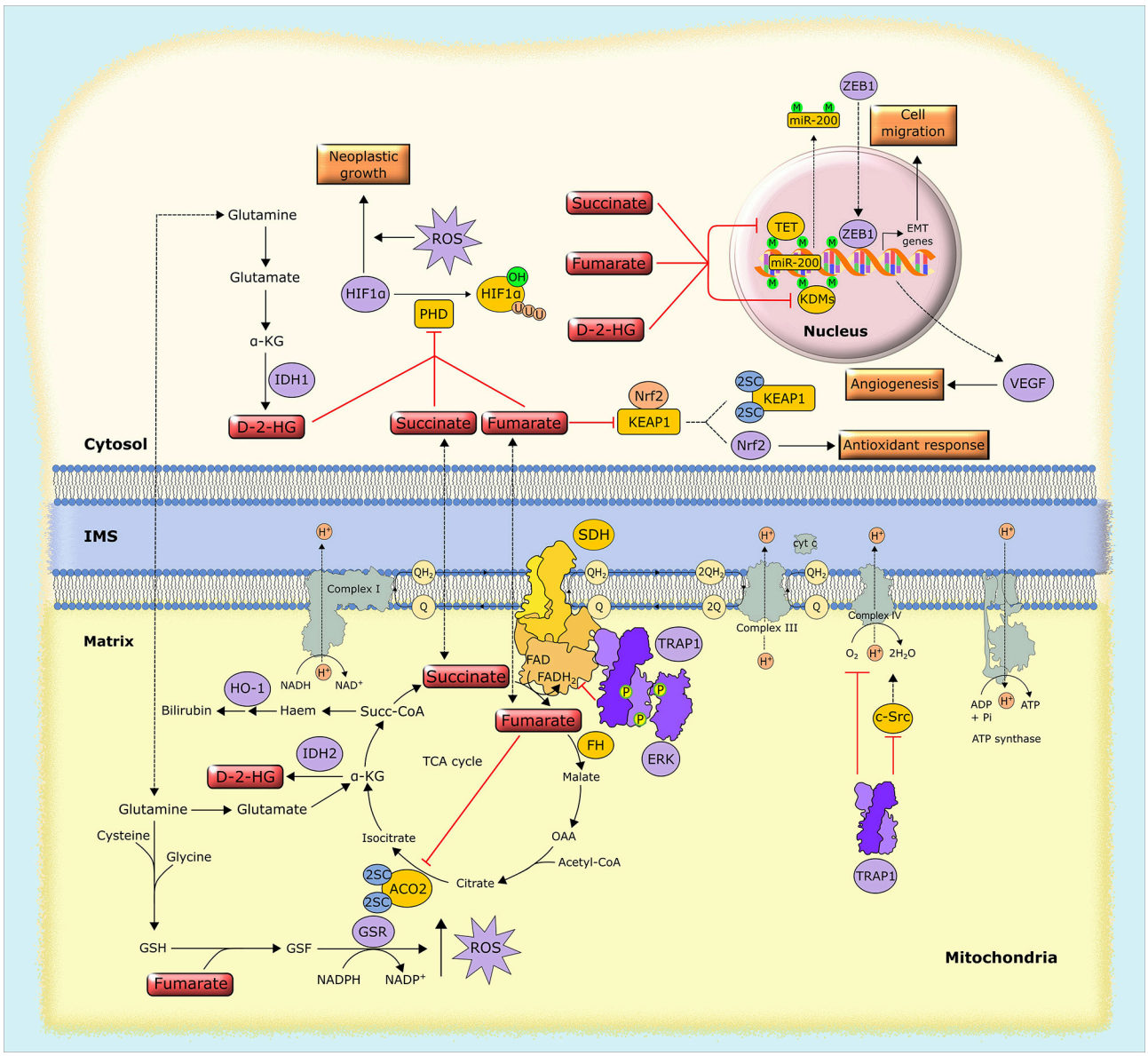


FIGURE 4 | Biochemical mechanisms of oncometabolite accumulation. Inactivating mutations in genes encoding succinate dehydrogenase (SDH) and fumarate hydratase (FH), as well as oncogenic mutations in isocytrate dehydrogenase (IDH), lead to the accumulation of succinate, fumarate and D-2-hydroxyglutarate (D-2-HG). These oncometabolites inhibit α-KG-dependent dioxygenases, including prolyl hydroxylases (PHDs), the ten-eleven translocation (TET) family of methylcytosine hydroxylases and histone lysine demethylases (KDMs), leading to HIF1α stabilization and alterations in gene expression through epigenetic modifications. Separately, fumarate inactivates by succination both aconitase 2 (ACO2) and Kelch-like ECH associated protein 1 (KEAP1), which results in the activation of the antioxidant pathway mediated by NRF2, and also generates succinated glutathione (GSF), an alternative substrate to glutathione reductase (GSR). Additionally, tumor cells can use alternative ways to increase oncometabolite concentration, such as the upregulation of the mitochondrial chaperone TRAP1, which inhibits the activity of SDH, leading to the intracellular accumulation of succinate. 2SC, succination of cysteine residues; EMT, epithelial-to-mesenchymal transition; GSH, reduced glutathione; HO-1, heme oxygenase-1; IMS, intermembrane space, M, methylation; OAA, oxaloacetate; P, phosphorylation, Succ-CoA, succinyl-CoA; U, ubiquitination.

More recently a third oncometabolite was identified, D-2-hydroxyglutarate (D-2-HG). D-2-HG accumulates to millimolar concentrations in tumors with monoallelic mutations in IDH1 and IDH2, $NADP^+$-dependent homodimers localized in cytoplasm and mitochondria, respectively, that convert isocitrate to α-KG (**Figure 4**). Mutant IDH1/2 dimerize with the wild-type protein and the heterodimeric enzyme acquires a neomorphic activity: the reduction of α-KG to D-2-HG in the presence of NADPH (128). As for fumarate and succinate, D-2-HG has a molecular structure that is similar to α-KG. Thus, D-2-HG acts as a competitive inhibitor of α-KG on the activity of α-KG-dependent dioxygenases (129). This results in global remodeling

of DNA methylome, with an increase in methylation of both CpG islands and histones (130), and in inhibition of the PHDs that degrade the HIFs (131). Mutant versions of cytoplasmic and mitochondrial IDH isoforms are present in a fraction of acute myeloid leukemias and in the majority of glioblastomas, as well as in chondrosarcomas and cholangiocarcinomas (131). Cells harboring high levels of D-2-HG are endowed with glutamate depletion, probably because glutamate is utilized to produce α-KG and subsequently converted to D-2-HG. Both low glutamate levels and a higher $NADP^+$/NADPH ratio caused by increased consumption of NADPH could suppress glutathione synthesis and regeneration, affecting the redox equilibrium of IDH mutant cells (9, 15, 24).

In addition to genetic mutations that affect the activity of enzymes involved in raising levels or generating oncometabolites, it is possible that tumor cells utilize more subtle ways to rapidly tune oncometabolite concentration, in order to afford rapid and flexible response to environmental changes. The molecular chaperone TRAP1 provides such an example. TRAP1 is a component of the HSP90 chaperone family whose expression is restricted to mitochondria and is increased in many malignancies (132). TRAP1 down-regulates the activity of both SDH (133) and respiratory complex IV (134), thus decreasing oxygen-coupled ATP synthesis and shifting the burden of ATP production to glycolysis (**Figure 4**). Inhibition of SDH by TRAP1 leads to succinate accumulation (133). Such an inhibition is further enhanced when ERK phosphorylates TRAP1, as in cells lacking the Ras GTPase-activating protein neurofibromin (100) that are characterized by deregulated induction of the Ras/ERK1/2 signaling pathway and form tumors in patients with the genetic syndrome neurofibromatosis type I (135). An increase in succinate levels induced by TRAP1 drives HIF1α stabilization independently of oxygen levels (136), *i.e.,* it generates conditions of pseudohypoxia, an adaptive feature of many tumors that allow them sustaining the neoplastic process even before hypoxic conditions are encountered by the growing malignancy. The importance of providing such a metabolic adaptableness is highlighted by the observation that abrogating TRAP1 expression ablates tumorigenicity when different cell types are xenografted in mice (100, 133), even though the specific importance of TRAP1 in neoplastic growth could be context-dependent (132).

REDOX HOMEOSTASIS AND MITOCHONDRIAL METABOLISM IN TUMORS

Mitochondria are the major source of intracellular reactive oxygen species (ROS), as about 1% of O_2 consumed by OXPHOS undergoes a one-electron reduction that forms a superoxide anion (137, 138). In addition, other mitochondrial metabolic enzymes, such as αKG dehydrogenase, PDH, the mitochondrial form of glycerol-3-phosphate dehydrogenase and acyl-CoA dehydrogenase can generate ROS (139–141). An excessive oxidant challenge damages biomolecules and leads to DNA mutations that eventually prompt cell senescence or death (142), but maintenance of a physiological redox equilibrium, or oxidative eustress (141), governs a variety of life processes and signal transduction pathways (137) (**Figure 5**).

Intracellular ROS levels are in general higher in tumor cells than in their non-transformed counterparts, and are involved in oncogene activation, tumor suppressor loss, metabolic rewiring, mutations in mitochondrial DNA (mtDNA) or hypoxia (142). ROS can reversibly target cysteine residues within the enzymatic sites of many phosphatases, such as the PI3K inhibitor PTEN, MAPK phosphatases and Tyr phosphatases (143), causing their inactivation. The consequent boost of kinase signaling pathways (144) affects mitochondrial metabolism, e.g. by tyrosine kinases that inhibit at multiple levels the PDC (145). In addition, many mitochondrial FAO enzymes contain ROS-sensitive Cys residues (**Figure 5**). Taken together these observations suggest that oxidative stress can tune mitochondrial metabolism by compromising both beta-oxidation of lipids and pyruvate entry into the TCA cycle (139). mtDNA mutations deregulate redox equilibrium by hampering respiration. Such mutations prompt *in vitro* and *in vivo* tumorigenicity, correlate with acquisition of metastatic potential and poor prognosis and can be used for cancer detection and determination of the degree of malignancy (20).

Hypoxia increases superoxide release from respiratory complex III, leading to PHD inhibition, possibly via oxidation of Fe^{2+} that is required for PHD function, and to the ensuing stabilization of HIFα subunits (52). In turn, HIF activation decreases ROS production by (i) down-modulating OXPHOS activity, as it suppresses SDHB expression (146), induces NDUFA4L2 (NADH dehydrogenase 1 subcomplex, 4-like 2), which attenuates complex I activity (147), and prompts the substitution of the COX subunit 4-1 with COX4-2, hence optimizing COX activity in low oxygen conditions (148); (ii) up-regulating miR-210, which orchestrates inhibition of mitochondrial bioenergetics by targeting the SDHD transcript and by repressing the iron–sulfur cluster assembly proteins that are required for the incorporation of [4Fe-4S] and [2Fe-2S] groups in respiratory complexes I, II and III; (iii) down-regulating mitochondrial biogenesis via c-Myc inhibition (147); (iv) inducing mitophagy through BNIP3, Bcl-2 and BN67IP3L/NIX induction (149). In keeping with this last point, absence of the mitophagy inducer Parkin enhances ROS generation by the persistence of dysfunctional mitochondria and increases tumorigenesis in multiple cancer models (150).

If activation of mitochondrial ROS generation remains below what triggers manifest cellular damage, it can contribute to the neoplastic process by causing DNA damage and genomic instability or by prompting dysregulated activation of crucial signaling pathways, eventually impacting on cell proliferation, angiogenesis and invasiveness (143). As an example, anchorage-independent growth of K-Ras-transformed cells requires an increase in mitochondrial ROS generated by respiratory complex III (105). Therefore, neoplastic cells must enhance their antioxidant devices, such as the tripeptide glutathione (l-glutamyl-l-cysteinyl-glycine), in order not to reach a threshold of oxidative damage incompatible with their survival. Both glutamine-derived glutamate and glucose-derived glycine are

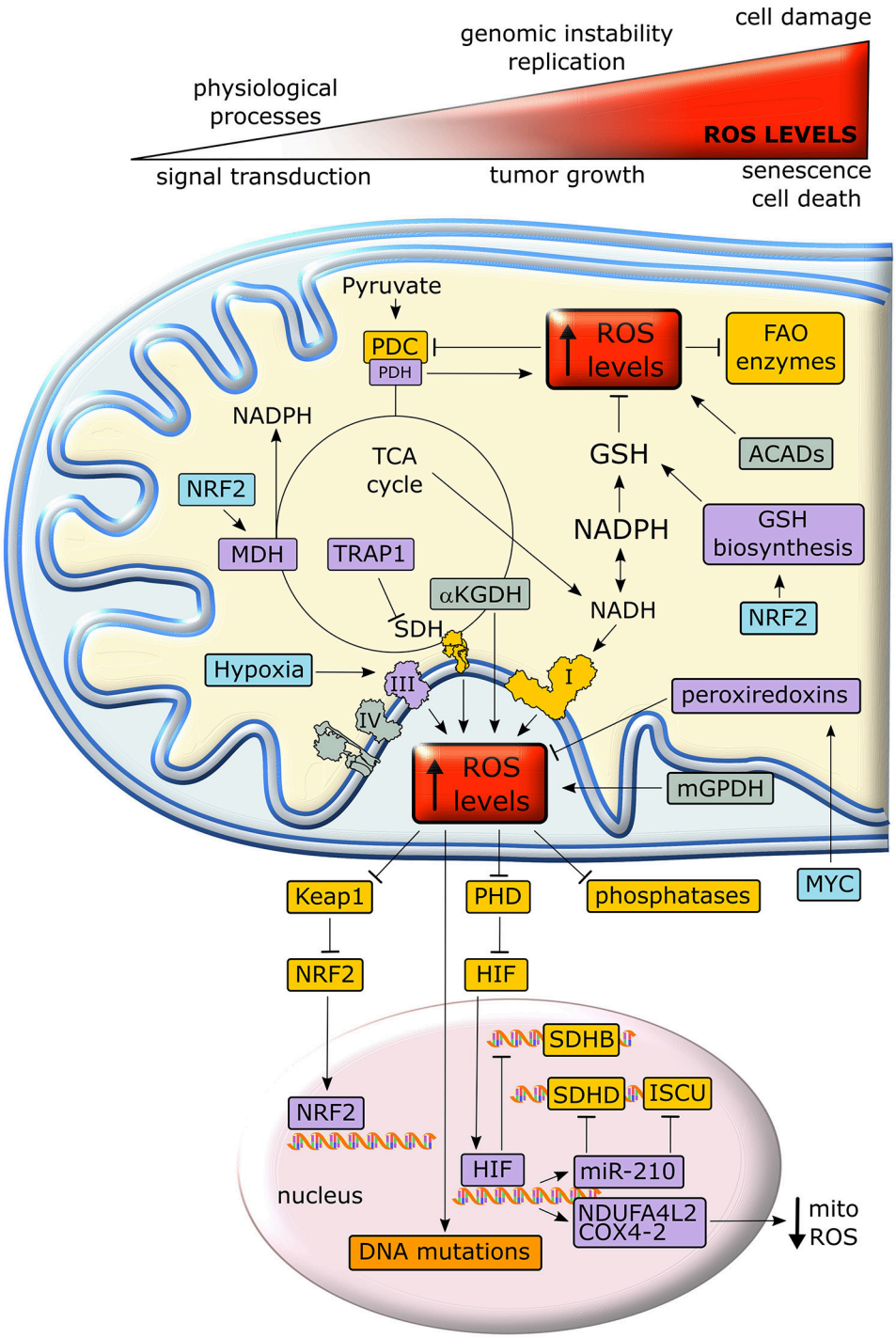


FIGURE 5 | Crosstalk between metabolism and redox homeostasis in cancer cell mitochondria. Neoplastic cells are characterized by high levels of mitochondrial ROS. Under a certain threshold, ROS facilitate tumor growth, but their excessive rise elicits oxidative damage and cell death. In cancer cell mitochondria, ROS levels are increased by respiratory chain complexes, αKGDH, mGPDH and ACADs and inhibit key enzymes of lipid metabolism and TCA cycle. In turn, ROS stimulate antioxidant defenses through stabilization of the transcription factors NRF2 and HIF1α. Proteins overexpressed or activated in cancer cells are indicated in purple, whereas proteins whose activity is down-regulated are shown in yellow. GSH, reduced glutathione; PDC, pyruvate dehydrogenase complex; PDH, pyruvate dehydrogenase; αKGDH, alpha-ketoglutarate dehydrogenase; SDH, succinate dehydrogenase; SDHB, succinate dehydrogenase subunit B; SDHD, succinate dehydrogenase subunit D; MDH, malate dehydrogenase; TRAP1, TNF receptor-associated protein 1; FAO, fatty acid oxidation; ACAD, acyl-CoA dehydrogenase; mGPDH, mitochondrial glycerol-3-phosphate dehydrogenase; NRF2, nuclear factor-E2-related factor 2; PHD prolyl hydroxilase; Keap1, Kelch-like ECH-associated protein 1; NDUFA4L2, NADH dehydrogenase 1 alpha subcomplex, 4-like 2; COX4-2, Cytochrome c OXidase subunit 4 isoform 2; ISCU, Iron–Sulfur cluster assembly proteins.

substrates for glutathione biosynthesis. NADPH, which is essential for the regeneration of reduced glutathione, is similarly obtained either by glucose through PPP and serine metabolism, or by glutamine via ME. Other anti-oxidant systems such as peroxiredoxins, which are induced by MYC, are also highly expressed in many cancer types (139).

The transcription factor Nrf2 (nuclear factor-E2-related factor 2) is a master regulator of cell response to oxidants that undergoes proteasomal degradation following ubiquitination by KEAP1. Under oxidative stress KEAP1 is inactivated, thus allowing nuclear accumulation and activation of Nrf2 (141) (**Figure 5**). Nrf2 induces enzymes that enhance carbon flux from glutamine toward GSH biosynthesis, utilization and regeneration, and stimulates NADPH production, e.g., by controlling ME, which boosts the oxidative decarboxylation of malate to pyruvate in order to feed the TCA cycle (142). Activation of Nrf2 increases mitochondrial membrane potential, FAO, ATP levels, rate of respiration and efficiency of oxidative phosphorylation (151). These protective functions against oxidative insults suggest that Nrf2 acts as a tumor suppressor, and indeed Nrf2 activation is beneficial in cancer chemoprevention and Nrf2-deficient mice are more sensitive to chemical carcinogenesis; in addition, the absence of Nrf2 has been related to a high metastatic potential (152). Nonetheless, the role of Nrf2 on tumorigenesis is highly contingent, as Nrf2 knockout mice are protected against tumor formation in the stomach, bladder, and skin (153) and activation of the Nrf2/KEAP1 system by somatic mutations is associated with a poor prognosis in patients (152) and has been observed in several cancer types. For instance, induction of the Nrf2/KEAP1 pathway occurs in the very early steps of hepatocarcinogenesis in the resistant hepatocyte rat model, where it associates with a metabolic rewiring toward increased glucose utilization, PPP activation and OXPHOS inhibition (154). In this model also the mitochondrial chaperone TRAP1 is highly expressed from the initial, pre-neoplastic lesions (154), and it probably contributes to the anti-oxidant mechanisms of tumor cells by decreasing SDH-generated ROS (155). TRAP1 has an oncogenic activity and its expression is induced in a variety of tumor types; however, TRAP1 levels decrease in the advanced stages of a small set of epithelial cancers (132). These contrasting observations on the role of Nrf2 and TRAP1 in neoplastic progression suggest that changes in cell redox equilibrium might have different effects on tumorigenesis, probably depending on tumor type and stage.

MITOCHONDRIAL Ca^{2+} AND METABOLIC PLASTICITY

Calcium ions are intracellular second messengers that tune a variety of fundamental cell processes (156). Mitochondria can accumulate high amounts of Ca^{2+}, thus acting both as Ca^{2+} stores that control the spatial and temporal shape of Ca^{2+}-mediated cellular signals, and as effectors that utilize Ca^{2+} to regulate cell survival, proliferation, redox state and metabolic changes. Mitochondrial Ca^{2+} homeostasis requires an efficient interplay between endoplasmic reticulum (ER), where most intracellular Ca^{2+} is stocked, and mitochondria in specialized microdomains called MAMs (Mitochondria-Associated Membranes) (157). In MAMs, Ca^{2+} is released from ER through IP3Rs (Inositol 1,4,5-triPhosphate Receptors) and is taken up by Mitochondrial Calcium Uniporter (MCU) complex, thus increasing Ca^{2+} concentration in mitochondrial matrix (158) (**Figure 6**).

Mitochondrial Ca^{2+} homeostasis is dysregulated in most neoplastic cells and contributes to their adaptations to stressful conditions in a fast and flexible way. Transduction pathways deregulated in cancer, such as PI3K/Akt or Ras signaling, can limit Ca^{2+} flux to mitochondria inhibiting IP3Rs, whereas tumor suppressors such as PTEN, BRCA1 or PML favor Ca^{2+} release from IP3Rs and the subsequent increase in mitochondrial Ca^{2+} levels (159, 160). Oncogenic mutations in p53 decrease the activity of SERCA (sarco/endoplasmic reticulum Ca^{2+}-ATPase), which takes Ca^{2+} up in ER, thus enhancing Ca^{2+} transfer to mitochondria in MAMs (160). Rapid spikes of Ca^{2+} levels in mitochondrial matrix induce the permeability transition pore (PTP), a mega-channel formed by ATP synthase whose prolonged opening elicits a sudden cell death (161). Thus, by slowing-down mitochondrial Ca^{2+} entry through MAMs, tumor cells can avoid to succumb to several noxious stimuli (162, 163). Hence, a fine tuning of IP3Rs activity is crucial in preventing lethal matrix Ca^{2+} overload. A complex interplay exists between IP3R regulation and Ca^{2+} homeostasis in mitochondria in tumors. Indeed, inhibition of Ca^{2+} transfer from ER to mitochondria decreases the viability of tumor cells compromising their bioenergetics. Notably, in these conditions neoplastic cells activate autophagy as a salvage mechanism, but this turns out to be insufficient for their survival (164).

More controlled raises in matrix Ca^{2+} concentration have important metabolic effects, as Ca^{2+} enhances the activity of mitochondrial dehydrogenases of the TCA cycle, IDH and αKGDH, and of PDH (165). These dehydrogenase reactions lead to formation of NADH that carries the reducing equivalents required for OXPHOS activity (158). Therefore, mitochondrial Ca^{2+} stimulates respiration and increases ROS generation (166). Moreover, Ca^{2+} stimulates the aspartate/glutamate exchanger in the inner mitochondrial membrane (167), further boosting TCA cycle activity by increasing matrix glutamate levels. Taken together, these observations suggest that lowering mitochondrial Ca^{2+} concentration could play a key role in maintaining a "Warburg-like" phenotype in neoplastic cells, while protecting them from PTP opening. However, the few studies that have directly assessed the role of mitochondrial Ca^{2+} in the tumorigenic process sketch elements of a more complex picture. For instance, the expression of MCU, whose activity can sharply increase mitochondrial Ca^{2+} concentration (158), is unexpectedly increased and associated to poor prognosis, invasiveness and metastasis in models of breast cancer and hepatocellular carcinoma (168, 169). Indeed, these observations directly link MCU activity to the maintenance of redox homeostasis. MCU silencing in triple negative breast cancer models decreases ROS levels by lowering ATP production and NADH cellular content. This hampers HIF-1α stability and transcriptional activity, decreasing cell motility and invasiveness, tumor growth, lymph node infiltration

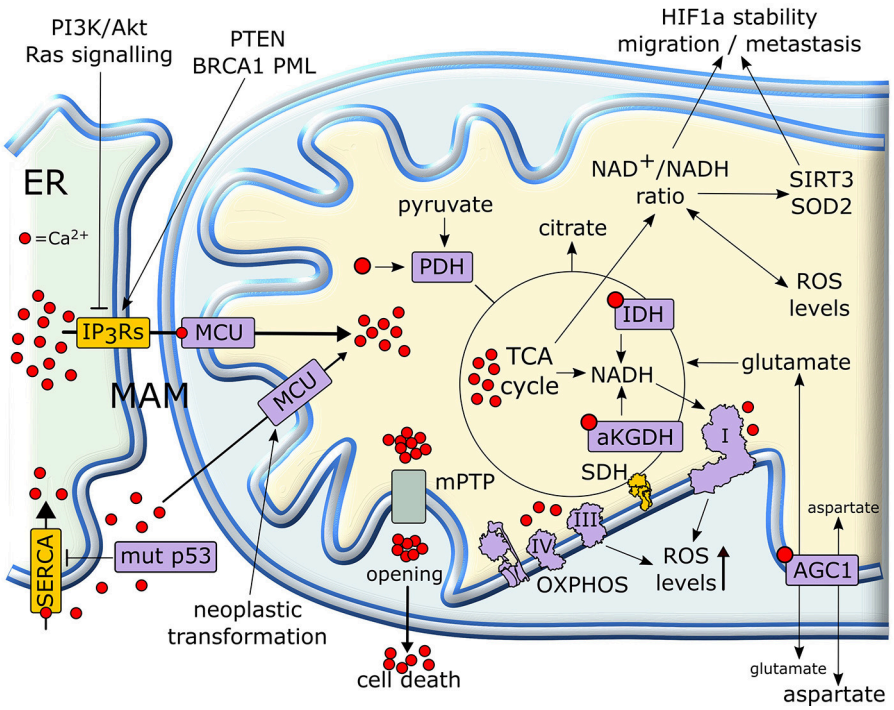


FIGURE 6 | Mitochondrial Ca^{2+} in the regulation of tumor cell metabolism. Ca^{2+} released from (ER) is taken up by mitochondria, where it increases the activity of TCA cycle and OXPHOS. In cancer cells, a rise in matrix Ca^{2+} can stimulate production of metabolic intermediates, glutamate transport and NADH formation for antioxidants defenses. NADH formation can also influence the pro-neoplastic stabilization of HIF1α. Several proteins with pro- or anti-neoplastic activity regulate IP3R Ca^{2+} channels in MAMs in order to prevent matrix Ca^{2+} overload, mPTP opening and the consequent cell death. Proteins overexpressed or activated in cancer cells are indicated in purple, whereas proteins whose activity is down-regulated are shown in yellow. ER, endoplasmic reticulum; IP3R, inositol 1,4,5-triphospate receptor; MCU, mitochondrial calcium uniporter; mPTP, mitochondrial permeability transition pore; PDH, pyruvate dehydrogenase; SDH, succinate dehydrogenase; αKGDH, alpha-ketoglutarate dehydrogenase; IDH, isocitrate dehydrogenase; HIF, hypoxia-inducible factor; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase.

and lung metastasis (169). In HCC cells, a MCU-dependent increase in matrix Ca^{2+} concentration stimulates TCA cycle activity and augments NADH/NAD^+ ratio. This inhibits a Sirtuin3/superoxide dismutase 2 axis that boosts mitochondrial ROS levels, which in turn sustain invasion and metastasis of hepatocellular carcinoma cells in an *in vivo* xenograft model (168).

Further studies are clearly needed to dissect how frequencies and amplitudes of mitochondrial Ca^{2+} oscillations influence the metabolic changes that characterize tumorigenesis.

POST TRANSLATIONAL REGULATION IN CANCER METABOLISM

Mitochondria can utilize post-translational modifications (PTMs) of their proteins in order to harmonize their activity to environmental conditions. A wide assortment of PTMs can lead to conformational changes in the tertiary structure of mitochondrial proteins, tuning their activity in response to changes in nutrient availability or redox conditions (170, 171) and furnishing cancer cells with a broad array of accurate and rapid metabolic adaptations (171). The investigation of these regulatory networks, and the functional connection with the metabolic changes that characterize neoplastic cells is complex and still in its infancy, and we will provide here only some general information.

The most prevalent mitochondrial PTM is acetylation of lysine residues, presumably because it requires acetyl-CoA that is highly compartmentalized in mitochondria (172). About 30% of mitochondrial proteins can undergo reversible acetylation. In general, this is an inhibitory mark for metabolic enzymes, as it would serve to sense the overproduction of acetyl-CoA, thus providing a negative feedback to mitochondrial metabolic circuitries that operate in an oxidative mode (173). Notably, hyperacetylation of mitochondrial proteins is observed in many diseases, including cancer (170). Acetylation is determined by the balance between the activity of acetyltransferases and deacetylases. Little is known on mitochondrial acetyltransferases. The only candidate is GCN5L1, which does not contain an acetyltransferase catalytic domain but promotes protein acetylation in the presence of acetyl-CoA, and its genetic disruption down-regulates acetylation of mitochondrial proteins. GCN5L1 is involved in lipid metabolism, as its induction promotes FAO, even if no data on tumor models are at present available (174).

Deacetylation is carried out by a class of enzymes called sirtuins, a protein family composed by 7 members, three of which (SIRT3–5) have a mitochondrial localization. Recent work has demonstrated that sirtuins are indeed deacylases, as they are able to transfer a variety of long acyl moieties including succinyl, malonyl, ADP-ribosyl and lipoyl groups, in addition to perform deacetylase reactions. All these reactions require NAD^+, thus linking sirtuin enzymatic activity to the metabolic state of the cell and poising them as metabolic stress sensors (175). Mitochondrial sirtuins orchestrate the coordinated regulation of substrate clusters, in order to efficiently tackle conditions of metabolic stress. SIRT3 is the major mitochondrial deacetylase and is activated upon starvation and by increased NAD^+ levels. In these conditions, SIRT3 enhances oxidative metabolism of fatty acids, by activating LCAD, and of amino acids, by increasing the activity of GDH and GLS2 (176, 177). SIRT3 also activates the PDH complex (PDC), thus promoting the conversion of pyruvate to acetyl-CoA (178). In parallel, SIRT3 stimulates ROS-mitigating systems such as IDH2, a TCA cycle enzyme that generates NADPH required to reduce glutathione (175), and superoxide dismutase 2, which converts superoxide to hydrogen peroxide that is then neutralized by glutathione (173). In addition, SIRT3 activates by deacetylation all OXPHOS complexes, in particular complex I and SDHA, the entry point of electrons from NADH and FADH2, respectively, thus promoting an efficient respiration (175). Taken together, these observations indicate that SIRT3 opposes a Warburg-like metabolism, and cells lacking SIRT3 exhibit genomic instability and are prone to tumorigenesis in xenografts. Accordingly, Sirt3-knockout mice develop mammary tumors, and in many human cancer types SIRT3 is deleted or expressed at a very low level (178). Nonetheless, the functional connections between mitochondrial sirtuin activity and tumor growth are multifaceted and far from being fully understood. Indeed, SIRT4 seems to have a tumor-suppressor role similar to that of SIRT3, as SIRT4-null mice develop lung tumors, loss of SIRT4 accelerates tumor progression in a mouse Burkitt lymphoma model and SIRT4 expression is reduced in several types of human cancers. However, SIRT4 plays opposing roles to SIRT3 in the regulation of several metabolic pathways: it promotes lipogenesis and represses fatty acid oxidation by inhibiting malonyl-CoA decarboxylase (170), negatively regulates PDC and represses GDH (177, 178). SIRT5, the last mitochondrial sirtuin, primarily demalonylates and desuccinylates lysine residues in a NAD^+-dependent way. Its functions in the metabolic rewiring of tumor cells are poorly understood, but it might be involved in glutamine metabolism as it inhibits GLS (177) and in OXPHOS and TCA regulation, as it decreases SDH activity by targeting both SDHA and SDHB and it inhibits PDC (170). Notably, SIRT5 could act as an oncogene, as it is overexpressed and associated with poor prognosis in human lung cancer (178). Finally, a further layer of metabolic regulation could be provided by sirtuin-directed PTMs such as phosphorylations (176).

Reversible phosphorylation at serine, threonine or tyrosine residues is emerging as an important mechanism regulating several aspects of mitochondrial metabolism. For instance, the inhibitory phosphorylation of PDC via enhanced expression of pyruvate dehydrogenase kinase-1 contributes to aerobic glycolysis and malignant phenotype, whereas PDH phosphatase exerts the opposite effect (145).

Adaptations to changes in nutrients and oxygen supply require a rapid OXPHOS regulation that can be achieved via reversible phosphorylations. The mitochondrial fraction of protein kinase A (PKA) activates in response to CO_2 generated in the TCA cycle. PKA increases the activity of respiratory complex I through phosphorylation of its NDUFS4 subunit (179). PKA also phosphorylates a subunit of cytochrome oxidase, preventing its allosteric inhibition by ATP and acting as a metabolic sensor to match OXPHOS activity with substrate availability and energy consumption requirements (180). Several phosphorylation sites are present on ATP synthase, but how they modulate the enzyme is still largely obscure. Preliminary data indicate that they could affect not only its activity, but also its assembly and dimerization (181). Activation of the tyrosine kinase Src increases the enzymatic activity of respiratory complex IV in isolated rat brain mitochondria (182); accordingly, the Src inhibitor dasatinib down-regulates the activity of respiratory complex IV in some tumor cell models, inhibiting their ROS-dependent invasiveness (134).

Tyrosine phosphorylation of SDH subunit A by the Src-like tyrosine kinase Fgr increases SDH activity, contributing to the capacity of mitochondria to modulate metabolism in conditions of nutrient restriction or hypoxia (183). Conversely, the kinase ERK1/2 decreases SDH activity. ERK1/2, SDH and the chaperone TRAP1 form a multimeric complex in mitochondria of neurofibromin-deficient cells. Mitochondrial ERK1/2 phosphorylates TRAP1, thus enhancing its inhibition of SDH. TRAP1 ablation or mutagenesis at the Ser residues targeted by ERK1/2 abrogates the tumorigenicity of cells lacking neurofibromin (100).

The mitochondrial fraction of the Ser/Thr kinase GSK-3 down-modulates the activity of PDH and of respiratory complex I (184). Moreover, GSK-3 phosphorylates the mitochondrial chaperone cyclophilin D (CyP-D), the best characterized proteinaceous regulator of the PTP, enhancing CyP-D-dependent PTP induction. In tumor cells, mitochondrial ERK1/2 inhibits by phosphorylation GSK-3, thus antagonizing PTP opening and cell death (99). A complex array of PTMs, including acetylations and nitrosylations in addition to phosphorylation, affects CyP-D activity (162). These PTMs could subtly tune the bioenergetic status of neoplastic cells, as CyP-D binds and down-regulates the enzymatic activity of ATP synthase (161).

MITOCHONDRIAL DYNAMICS AND CANCER METABOLISM

Mitochondria are extremely dynamic organelles, undergoing the opposite processes of fusion and fission in a coordinated and balanced way. A comprehension of how mitochondrial dynamics contribute to the metabolic rewiring of cancer cells is still in its infancy, but several evidences are emerging that link decreased fusion and enhanced fission to neoplastic transformation, invasion and metastasis (185, 186). Signaling via oncogenic

MAPK and PI3K promotes fission (187), and in certain tumor settings high levels of the fission protein DRP1, whose activity is increased by ERK-dependent phosphorylation, negatively correlate with survival of patients. Conversely, overexpression of the fusion proteins mitofusins decreases tumor growth, and their levels are directly related to OXPHOS activity and ATP production in several cell models (188).

However, the assumption that glycolytic cells have fragmented mitochondria, whereas OXPHOS is increased in cells with elongated mitochondria, appears as an oversimplification (188). Indeed, a prolonged DRP1 downregulation can inhibit respiration, suggesting that a proper OXPHOS modulation requires a balanced interplay between mitochondrial fission and fusion (189). Thus, it is difficult at present to make mechanistic correlations between mitochondrial dynamics and metabolism and to understand whether changes in the mitochondrial network of cancer cells are priming events or consequences of their metabolic rewiring. For instance, even if activation of several oncogenes increases fission, some others, such as Myc, promote mitochondrial fusion (187). Moreover, mitochondrial shape can impact on intramitochondrial Ca^{2+} waves (185) and on Ca^{2+} fluxes at MAMs (189), thus playing a complex and probably context-dependent role on the metabolic adaptations of tumor cells (see section Mitochondrial Ca^{2+} And Metabolic Plasticity).

Mitochondrial fission is strictly connected to mitophagy, a quality control process that maintains mitochondrial integrity and function through removal of damaged organelles, which must be isolated from the healthy network via DRP1-dependent sequestration (189, 190). Mitophagy is activated by a variety of stresses usually encountered by neoplastic cells, including hypoxia, nutrient deprivation, DNA damage and inflammation, which eventually cause mitochondrial membrane depolarization and decline in respiratory capability (149). Therefore, any impairment in the mitophagy process leads to accumulation of dysfunctional mitochondria, hence decreasing respiration and ATP production and increasing ROS levels. In general, defects in mitophagy affect the metabolic plasticity of mitochondria in response to environmental stresses such as altered Ca^{2+} signaling, ROS generation and changes in nutrient availability, further amplifying their noxious effects on the cell. In cancer, a disruption of the homeostatic equilibrium between mitophagy and mitogenesis occurs (191, 192). It has been proposed that impairment of a correct mitophagy could be advantageous for the early phases of neoplastic growth, contributing to set a novel redox equilibrium, whereas later stages of tumor progression would be favored by mitophagy, as it would protect tumor cells from excessive mitochondrial damage, surge in ROS levels and apoptosis (189). Further work is certainly needed to draw a more complete picture of the functional interplay between mitochondrial fusion and fission, mitophagy and metabolic rewiring of cancer cells.

CONCLUDING REMARKS

Metabolism is a multilevel process, encompassing and integrating a myriad of factors both at the organismal scale, such as age or lifestyle, and at the local level, including cellular composition of the microenvironment, nutrient supply and stiffness of the extracellular matrix. Accordingly, aberrant metabolic reprogramming in cancer is both the cause and the effect of alterations at multiple levels that reverberate on each other.

The (epi)genomic landscape of neoplastic cells, the intertwined molecular signaling between immune, stromal and other non-transformed cells with the malignant ones in tumor microenvironment (193, 194), as well as increases in hydrostatic forces and in the stiffness of the tumor milieu (195), are all factors that constantly tune cancer cell metabolism to fluctuating environmental conditions, leading to metabolic heterogeneity also across different areas of the same tumor. In neoplastic cells, mitochondria constitute a point of integration for many of these metabolic circuitries. For instance, reciprocal feedbacks exist between mechanosignaling, the process by which cells convert extracellular mechanical forces into biochemical outputs, and glutamine metabolism, as glutamine partly controls focal adhesions and actin stress fiber assembly, and in turn stiffness changes glutamine fluxes (196).

Moreover, mitochondria are at the heart of mechanisms that balance a variety of intracellular metabolic circuitries. For instance, NADPH homeostasis is maintained by several mitochondrial metabolic circuitries via TCA cycle intermediates and ATP generated by OXPHOS. These pathways include one carbon metabolism, PPP, whose oxidative branch is enhanced by citrate- or ATP-dependent inhibition of late glycolytic steps (5, 11), glutamine-derived carbons diverted out of the TCA cycle to convert malate into pyruvate via ME, and IDH (6, 197, 198). p53 represses the transcription of ME genes, thus inhibiting the usage of TCA cycle intermediates for NADPH production (68). Conversely, citrate inhibits PFK, pyruvate kinase and PDH, blocking pyruvate generation from glycolysis and leading to an increase in ME activity to maintain pyruvate levels, but also to provide NADPH (11). Another important source of NADPH is lipid β-oxidation, which becomes a major fuel for ATP synthesis during invasion and metastasis (199), further highlighting the tight connection between metabolic adaptations and biological conditions in cancer cells.

Citrate and acetyl-CoA provide other examples of multiple metabolic intersections. Mitochondrial citrate is a TCA cycle metabolite that originates by the condensation of OAA and acetyl-CoA, but it also forms via glutamine-fueled reductive carboxylation (47, 200), which is particularly important under hypoxic conditions and supports anchorage-independent growth of neoplastic cells by mitigating oxidative stress through a coordinated regulation of NADH/NADPH dependent IDH1 and 2 in cytosol and mitochondria (201). In mitochondria, citrate down-regulates SDH (11), *i.e.,* the point of integration between TCA cycle and OXPHOS, contributing to raise the levels of the oncometabolite succinate. Alternatively, citrate can move to cytosol, where it both favors glucose usage in PPP and serine synthesis by inhibiting the late glycolytic steps and is converted into acetyl-CoA (11). In turn, acetyl-CoA starts lipid synthesis and sustains acetylation reactions (see section Post Translational Regulation In Cancer Metabolism)

and influences a variety of biochemical circuitries involved in the neoplastic process, including histone acetylation and chromatin remodeling as well as redox homeostasis via acetylation of superoxide dismutase and IDH (1). Regulation of histone acetylation showcases the fundamental role played by metabolic enzymes and metabolites in gene expression control (117). One interesting example is provided by the PDC, which locates both in mitochondria and nucleus of prostate cancer cells. This compartmentalization is instrumental to orchestrate lipid biosynthesis both by providing cytosolic citrate and by inducing the transcription of genes for lipid synthesis by regulating histone acetylation (202).

As described in section Mutations Of Mitochondrial Enzymes In Cancer Metabolism, tumors with mutations in enzymes of the TCA cycle constitute an excellent model to study how mitochondrial metabolism causes pro-neoplastic (epi)genomic changes (203), and they also provide clues to identify molecular vulnerabilities that can be exploited for anti-tumor strategies. For instance, pyruvate carboxylase (PC) enables aspartate synthesis in SDH-deficient tumor cells, creating a metabolic vulnerability. lack of SDH activity commits cells to consume extracellular pyruvate, which sustains Warburg-like bioenergetic features. Pyruvate carboxylation diverts glucose-derived carbons into aspartate biosynthesis, thus sustaining cell growth (204, 205).

Further layers of complexity are provided by the heterogeneity of cellular components of the tumor mass. For instance, cancer stem cells (CSC) constitute a small population of self-renewal neoplastic cells that are *per se* capable of promoting tumor growth. The metabolic features of CSC differ from those of the bulky neoplasm, and recent evidences point toward an OXPHOS phenotype in CSC. In a sort of reverse Warburg metabolism, OXPHOS CSC might be fed by glycolytic tumor cells or by cells of the tumor microenvironment, such as cancer associated fibroblasts, and could switch to a glycolytic metabolism under hypoxia (206). Examples of symbiotic nutrient sharing between neoplastic cells and tumor microenvironment are observed in metastatic ovarian cancer cells, breast cancer cells or leukemic stem cells, which oxidize fatty acids supplied from surrounding adipocytes to sustain proliferation, survival and invasiveness and possibly to preserve cell redox balance (207, 208). Moreover, horizontal transfer of mitochondria from stromal to cancer cells can lead to an increase in OXPHOS metabolism of the latter (19, 209).

Metabolic plasticity of tumor cell mitochondria offers a high window of opportunity for efficient anti-cancer therapy, since transformed cells have metabolic needs that differ from their non-transformed counterparts, and molecules such PDH, IDH1/2 or glutaminase inhibitors are already in clinical trials. Nonetheless, such a plasticity can also be a hurdle when trying to develop selective therapeutic strategies. As an example, ovarian cancer cells gain resistance to antiangiogenic therapy by shifting their metabolic phenotype toward a highly glycolytic one (210). Importantly, tumor cell dependency on specific bioenergetic features *in vitro* can be extremely different from the *in vivo* situation, due to off-target effects, suboptimal pharmacokinetic properties of the compound or metabolic heterogeneity of the neoplastic mass.

A profound comprehension of the biochemical mechanisms that govern the bioenergetic flexibility of tumor cell mitochondria and its interplay with a multitude of extra-mitochondrial signals constitutes a central dowel to build an integrate model of the metabolic features that hallmark cancer. Incorporation of data obtained at different scales of analysis, from the organism to the organelle, remains a tremendous task. Nonetheless, huge advances have been recently made unveiling biochemical processes and therapeutic opportunities that were unimaginable even few years ago.

AUTHOR CONTRIBUTIONS

All authors contributed to writing the text and drawing the figures and approved the manuscript for publication.

ACKNOWLEDGMENTS

Research in the AR group is supported by grants from University of Padova, Children's Tumor Foundation Drug Discovery Initiative (grant 2016A-05-009), Neurofibromatosis Therapeutic Acceleration Program, Associazione Italiana Ricerca Cancro (AIRC grant IG 2017/20749), Piano for Life Onlus and Linfa Onlus. IM is supported by a post-doctoral Young Investigator Award Grant from the Children's Tumor Foundation.

REFERENCES

1. Vyas S, Zaganjor E, Haigis MC. Mitochondria and cancer. *Cell* (2016) 166:555–66. doi: 10.1016/j.cell.2016.07.002
2. Warburg O. On the origin of cancer cells. *Science* (1956) 123:309–14. doi: 10.1126/science.123.3191.309
3. Warburg O, Wind F, Negelein E. The metabolism of tumors in the body. *J Gen Physiol*. (1927) 8:519–30. doi: 10.1085/jgp.8.6.519
4. Ward PS, Thompson CB. Metabolic reprogramming: a cancer hallmark even Warburg did not anticipate. *Cancer Cell* (2012) 21:297–308. doi: 10.1016/j.ccr.2012.02.014
5. Patra KC, Hay N. The pentose phosphate pathway and cancer. *Trends Biochem Sci*. (2014) 39:347–54. doi: 10.1016/j.tibs.2014.06.005
6. Boroughs LK, DeBerardinis RJ. Metabolic pathways promoting cancer cell survival and growth. *Nat Cell Biol*. (2015) 17:351–9. doi: 10.1038/ncb3124
7. Dang CV. Links between metabolism and cancer. *Genes Dev*. (2012) 26:877–90. doi: 10.1101/gad.189365.112
8. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* (2009) 324:1029–33. doi: 10.1126/science.1160809
9. Altman BJ, Stine ZE, Dang CV. From Krebs to clinic: glutamine metabolism to cancer therapy. *Nat Rev Cancer* (2016) 16:749. doi: 10.1038/nrc.2016.114
10. Chiarugi A, Dolle C, Felici R, Ziegler M. The NAD metabolome–a key determinant of cancer cell biology. *Nat Rev Cancer* (2012) 12:741–52. doi: 10.1038/nrc3340
11. Icard P, Poulain L, Lincet H. Understanding the central role of citrate in the metabolism of cancer cells. *Biochim Biophys Acta* (2012) 1825:111–6. doi: 10.1016/j.bbcan.2011.10.007
12. Katsyuba E, Auwerx J. Modulating NAD(+) metabolism, from bench to bedside. *EMBO J* (2017) 36:2670–83. doi: 10.15252/embj.201797135

13. Rohrig F, Schulze A. The multifaceted roles of fatty acid synthesis in cancer. *Nat Rev Cancer* (2016) 16:732–49. doi: 10.1038/nrc.2016.89
14. Schulze A, Harris AL. How cancer metabolism is tuned for proliferation and vulnerable to disruption. *Nature* (2012) 491:364–73 doi: 10.1038/nature11706
15. Zhang J, Pavlova NN, Thompson CB. Cancer cell metabolism: the essential role of the nonessential amino acid, glutamine. *EMBO J*. (2017) 36:1302–15. doi: 10.15252/embj.201696151
16. Wallace DC. Mitochondria and cancer. *Nat Rev Cancer* (2012) 12:685–98. doi: 10.1038/nrc3365
17. De Luise M, Girolimetti G, Okere B, Porcelli AM, Kurelac I, Gasparre G. Molecular and metabolic features of oncocytomas: Seeking the blueprints of indolent cancers. *Biochim Biophys Acta* (2017) 1858:591–601. doi: 10.1016/j.bbabio.2017.01.009
18. Joshi S, Tolkunov D, Aviv H, Hakimi AA, Yao M, Hsieh JJ, et al. The genomic landscape of renal oncocytoma identifies a metabolic barrier to tumorigenesis. *Cell Rep*. (2015) 13:1895–908. doi: 10.1016/j.celrep.2015.10.059
19. Tan AS, Baty JW, Dong LF, Bezawork-Geleta A, Endaya B, Goodwin J, et al. Mitochondrial genome acquisition restores respiratory function and tumorigenic potential of cancer cells without mitochondrial DNA. *Cell Metab*. (2015) 21:81–94. doi: 10.1016/j.cmet.2014.12.003
20. Zong WX, Rabinowitz JD, White E. Mitochondria and cancer. *Mol Cell* (2016) 61:667–76. doi: 10.1016/j.molcel.2016.02.011
21. Frezza C. The role of mitochondria in the oncogenic signal transduction. *Int J Biochem Cell Biol*. (2014) 48C:11–7 doi: 10.1016/j.biocel.2013.12.013
22. Gaude E, Frezza C. Defects in mitochondrial metabolism and cancer. *Cancer Metab*. (2014) 2:10. doi: 10.1186/2049-3002-2-10
23. Adam J, Yang M, Soga T, Pollard PJ. Rare insights into cancer biology. *Oncogene* (2014) 33:2547–56. doi: 10.1038/onc.2013.222
24. Yang M, Soga T, Pollard PJ. Oncometabolites: linking altered metabolism with cancer. *J Clin Invest*. (2013) 123:3652–8. doi: 10.1172/JCI67228
25. Danhier P, Banski P, Payen VL, Grasso D, Ippolito L, Sonveaux P, et al. Cancer metabolism in space and time: beyond the Warburg effect. *Biochim Biophys Acta* (2017) 1858:556–72. doi: 10.1016/j.bbabio.2017.02.001
26. Chen JQ, Russo J. Dysregulation of glucose transport, glycolysis, TCA cycle and glutaminolysis by oncogenes and tumor suppressors in cancer cells. *Biochim Biophys Acta* (2012) 1826:370–84. doi: 10.1016/j.bbcan.2012.06.004
27. Tarrado-Castellarnau M, de Atauri P, Cascante M. Oncogenic regulation of tumor metabolic reprogramming. *Oncotarget* (2016) 7:62726–53. doi: 10.18632/oncotarget.10911
28. Keith B, Johnson RS, Simon MC. HIF1alpha and HIF2alpha: sibling rivalry in hypoxic tumour growth and progression. *Nat Rev Cancer* (2012) 12:9–22. doi: 10.1038/nrc3183
29. Koh MY, Powis G. Passing the baton: the HIF switch. *Trends Biochem Sci*. (2012) 37:364–72. doi: 10.1016/j.tibs.2012.06.004
30. Qiu GZ, Jin MZ, Dai JX, Sun W, Feng JH, Jin WL. Reprogramming of the tumor in the hypoxic niche: the emerging concept and associated therapeutic strategies. *Trends Pharmacol Sci*. (2017) 38:669–86. doi: 10.1016/j.tips.2017.05.002
31. Semenza GL. HIF-1 mediates metabolic responses to intratumoral hypoxia and oncogenic mutations. *J Clin Invest* (2013) 123:3664–71. doi: 10.1172/JCI67230
32. Mathupala SP, Ko YH, Pedersen PL. The pivotal roles of mitochondria in cancer: Warburg and beyond and encouraging prospects for effective therapies. *Biochim Biophys Acta* (2010) 1797:1225–30 doi: 10.1016/j.bbabio.2010.03.025
33. Roberts DJ, Miyamoto S. Hexokinase II integrates energy metabolism and cellular protection: akting on mitochondria and TORCing to autophagy. *Cell Death Differ* (2015) 22:248–57. doi: 10.1038/cdd.2014.173
34. Chiara F, Castellaro D, Marin O, Petronilli V, Brusilow WS, Juhaszova M, et al. Hexokinase II detachment from mitochondria triggers apoptosis through the permeability transition pore independent of voltage-dependent anion channels. *PLoS ONE* (2008) 3:e1852. doi: 10.1371/journal.pone.0001852
35. Masgras I, Rasola A, Bernardi P. Induction of the permeability transition pore in cells depleted of mitochondrial DNA. *Biochim Biophys Acta* (2012) 1817:1860–6. doi: 10.1016/j.bbabio.2012.02.022
36. Pantic B, Trevisan E, Citta A, Rigobello MP, Marin O, Bernardi P, et al. Myotonic dystrophy protein kinase (DMPK) prevents ROS-induced cell death by assembling a hexokinase II-Src complex on the mitochondrial surface. *Cell Death Dis*. (2013) 4:e858 doi: 10.1038/cddis.2013.385
37. Patra KC, Wang Q, Bhaskar PT, Miller L, Wang Z, Wheaton W, et al. Hexokinase 2 is required for tumor initiation and maintenance and its systemic deletion is therapeutic in mouse models of cancer. *Cancer Cell* (2013) 24:213–28. doi: 10.1016/j.ccr.2013.06.014
38. Roberts DJ, Tan-Sah VP, Ding EY, Smith JM, Miyamoto S. Hexokinase-II positively regulates glucose starvation-induced autophagy through TORC1 inhibition. *Mol Cell* (2014) 53:521–33. doi: 10.1016/j.molcel.2013.12.019
39. Kim JW, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab*. (2006) 3:177–85. doi: 10.1016/j.cmet.2006.02.002
40. Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. *Cell Metab*. (2006) 3:187–97. doi: 10.1016/j.cmet.2006.01.012
41. Greer SN, Metcalf JL, Wang Y, Ohh M. The updated biology of hypoxia-inducible factor. *EMBO J*. (2012) 31:2448–60. doi: 10.1038/emboj.2012.125
42. Cortes-Cros M, Hemmerlin C, Ferretti S, Zhang J, Gounarides JS, Yin H, et al. M2 isoform of pyruvate kinase is dispensable for tumor maintenance and growth. *Proc Natl Acad Sci USA*. (2013) 110:489–94. doi: 10.1073/pnas.1212780110
43. Vacanti NM, Divakaruni AS, Green CR, Parker SJ, Henry RR, Ciaraldi TP, et al. Regulation of substrate utilization by the mitochondrial pyruvate carrier. *Mol Cell* (2014) 56:425–35. doi: 10.1016/j.molcel.2014.09.024
44. Ackerman D, Simon MC. Hypoxia, lipids, and cancer: surviving the harsh tumor microenvironment. *Trends Cell Biol*. (2014) 24:472–8. doi: 10.1016/j.tcb.2014.06.001
45. Currie E, Schulze A, Zechner R, Walther TC, Farese RV, Jr. Cellular fatty acid metabolism and cancer. *Cell Metab*. (2013) 18:153–61. doi: 10.1016/j.cmet.2013.05.017
46. Masson N, Ratcliffe PJ. Hypoxia signaling pathways in cancer metabolism: the importance of co-selecting interconnected physiological pathways. *Cancer Metab*. (2014) 2:3. doi: 10.1186/2049-3002-2-3
47. Metallo CM, Gameiro PA, Bell EL, Mattaini KR, Yang J, Hiller K, et al. Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. *Nature* (2011) 481:380–4. doi: 10.1038/nature10602
48. Sun RC, Denko NC. Hypoxic regulation of glutamine metabolism through HIF1 and SIAH2 supports lipid synthesis that is necessary for tumor growth. *Cell Metab*. (2014) 19:285–92. doi: 10.1016/j.cmet.2013.11.022
49. Huang, Li T, Li X, Zhang L, Sun L, He X, et al. HIF-1-mediated suppression of acyl-CoA dehydrogenases and fatty acid oxidation is critical for cancer progression. *Cell Rep*. (2014) 8:1930–42. doi: 10.1016/j.celrep.2014.08.028
50. German NJ, Yoon H, Yusuf RZ, Murphy JP, Finley LW, Laurent G, et al. PHD3 loss in cancer enables metabolic reliance on fatty acid oxidation via deactivation of ACC2. *Mol Cell* (2016) 63:1006–20. doi: 10.1016/j.molcel.2016.08.014
51. LaGory EL, Wu C, Taniguchi CM, Ding CC, Chi JT, von Eyben R, et al. Suppression of PGC-1alpha is critical for reprogramming oxidative metabolism in renal cell carcinoma. *Cell Rep*. (2015) 12:116–27. doi: 10.1016/j.celrep.2015.06.006
52. LaGory EL, Giaccia AJ. The ever-expanding role of HIF in tumour and stromal biology. *Nat Cell Biol*. (2016) 18:356–65. doi: 10.1038/ncb3330
53. Dang CV. MYC on the path to cancer. *Cell* (2012) 149:22–35 doi: 10.1016/j.cell.2012.03.003
54. Dejure FR, Eilers M. MYC and tumor metabolism: chicken and egg. *EMBO J*. (2017) 36:3409–20. doi: 10.15252/embj.201796438
55. Stine ZE, Walton ZE, Altman BJ, Hsieh AL, Dang CV. MYC, metabolism, and cancer. *Cancer Discov*. (2015) 5:1024–39. doi: 10.1158/2159-8290.CD-15-0507
56. Dang CV. Rethinking the Warburg effect with Myc micromanaging glutamine metabolism. *Cancer Res*. (2010) 70:859–62. doi: 10.1158/0008-5472.CAN-09-3556
57. Ye J, Fan J, Venneti S, Wan YW, Pawel BR, Zhang J, et al. Serine catabolism regulates mitochondrial redox control during hypoxia. *Cancer Discov*. (2014) 4:1406–17. doi: 10.1158/2159-8290.CD-14-0250

58. Kim D, Fiske BP, Birsoy K, Freinkman E, Kami K, Possemato RL, et al. SHMT2 drives glioma cell survival in ischaemia but imposes a dependence on glycine clearance. *Nature* (2015) 520:363–7. doi: 10.1038/nature14363
59. Hensley CT, Wasti AT, DeBerardinis RJ. Glutamine and cancer: cell biology, physiology, and clinical opportunities. *J Clin Invest* (2013) 123:3678–84. doi: 10.1172/JCI69600
60. Rasmussen BB, Holmback UC, Volpi E, Morio-Liondore B, Paddon-Jones D, Wolfe RR. Malonyl coenzyme A and the regulation of functional carnitine palmitoyltransferase-1 activity and fat oxidation in human skeletal muscle. *J Clin Invest* (2002) 110:1687–93. doi: 10.1172/JCI0215715
61. Kastenhuber ER, Lowe SW. Putting p53 in context. *Cell* (2017) 170:1062–78. doi: 10.1016/j.cell.2017.08.028
62. Kruiswijk F, Labuschagne CF, Vousden KH. p53 in survival, death and metabolic health: a lifeguard with a licence to kill. *Nat Rev Mol Cell Biol*. (2015) 16:393–405. doi: 10.1038/nrm4007
63. Muller PA, Vousden KH. p53 mutations in cancer. *Nat Cell Biol*. (2013) 15:2–8. doi: 10.1038/ncb2641
64. Maiuri MC, Galluzzi L, Morselli E, Kepp O, Malik SA, Kroemer G. Autophagy regulation by p53. *Curr Opin Cell Biol*. (2010) 22:181–5. doi: 10.1016/j.ceb.2009.12.001
65. Vaseva AV, Marchenko ND, Ji K, Tsirka SE, Holzmann S, Moll UM. p53 opens the mitochondrial permeability transition pore to trigger necrosis. *Cell* (2012) 149:1536–48. doi: 10.1016/j.cell.2012.05.014
66. Saxton RA, Sabatini DM. mTOR signaling in growth, metabolism, and disease. *Cell* (2017) 168:960–76. doi: 10.1016/j.cell.2017.02.004
67. Garcia D, Shaw RJ. AMPK: mechanisms of cellular energy sensing and restoration of metabolic balance. *Mol Cell* (2017) 66:789–800. doi: 10.1016/j.molcel.2017.05.032
68. Berkers CR, Maddocks OD, Cheung EC, Mor I, Vousden KH. Metabolic regulation by p53 family members. *Cell Metab*. (2013) 18:617–33. doi: 10.1016/j.cmet.2013.06.019
69. Feng Z, Levine AJ. The regulation of energy metabolism and the IGF-1/mTOR pathways by the p53 protein. *Trends Cell Biol*. (2010) 20:427–34. doi: 10.1016/j.tcb.2010.03.004
70. Schwartzenberg-Bar-Yoseph F, Armoni M, Karnieli E. The tumor suppressor p53 down-regulates glucose transporters GLUT1 and GLUT4 gene expression. *Cancer Res*. (2004) 64:2627–33. doi: 10.1158/0008-5472.CAN-03-0846
71. Kondoh H, Lleonart ME, Gil J, Wang J, Degan P, Peters G, et al. Glycolytic enzymes can modulate cellular life span. *Cancer Res*. (2005) 65:177–85
72. Lee P, Vousden KH, Cheung EC. TIGAR, TIGAR, burning bright. *Cancer Metab*. (2014) 2:1 doi: 10.1186/2049-3002-2-1
73. Jiang P, Du W, Wang X, Mancuso A, Gao X, Wu M, et al. p53 regulates biosynthesis through direct inactivation of glucose-6-phosphate dehydrogenase. *Nat Cell Biol*. (2011) 13:310–6. doi: 10.1038/ncb2172
74. Hu W, Zhang C, Wu R, Sun Y, Levine A, Feng Z. Glutaminase 2, a novel p53 target gene regulating energy metabolism and antioxidant function. *Proc Natl Acad Sci USA*. (2010) 107:7455–60. doi: 10.1073/pnas.1001006107
75. Suzuki S, Tanaka T, Poyurovsky MV, Nagano H, Mayama T, Ohkubo S, et al. Phosphate-activated glutaminase (GLS2), a p53-inducible regulator of glutamine metabolism and reactive oxygen species. *Proc Natl Acad Sci USA*. (2010) 107:7461–6. doi: 10.1073/pnas.1002459107
76. Contractor T, Harris CR. p53 negatively regulates transcription of the pyruvate dehydrogenase kinase Pdk2. *Cancer Res*. (2012) 72:560–7. doi: 10.1158/0008-5472.CAN-11-1215
77. Sanchez-Macedo N, Feng J, Faubert B, Chang N, Elia A, Rushing EJ, et al. Depletion of the novel p53-target gene carnitine palmitoyltransferase 1C delays tumor growth in the neurofibromatosis type I tumor model. *Cell Death Differ* (2013) 20:659–68. doi: 10.1038/cdd.2012.168
78. Zaugg K, Yao Y, Reilly PT, Kannan K, Kiarash R, Mason J, et al. Carnitine palmitoyltransferase 1C promotes cell survival and tumor growth under conditions of metabolic stress. *Genes Dev*. (2011) 25:1041–51. doi: 10.1101/gad.1987211
79. Assaily W, Rubinger DA, Wheaton K, Lin Y, Ma W, Xuan W, et al. ROS-mediated p53 induction of Lpin1 regulates fatty acid oxidation in response to nutritional stress. *Mol Cell* (2011) 44:491–501. doi: 10.1016/j.molcel.2011.08.038
80. Wang SJ, Yu G, Jiang L, Li T, Lin Q, Tang Y, et al. p53-Dependent regulation of metabolic function through transcriptional activation of pantothenate kinase-1 gene. *Cell Cycle* (2013) 12:753–61. doi: 10.4161/cc.23597
81. Liu Y, He Y, Jin A, Tikunov AP, Zhou L, Tollini LA, et al. Ribosomal protein-Mdm2-p53 pathway coordinates nutrient stress with lipid metabolism by regulating MCD and promoting fatty acid oxidation. *Proc Natl Acad Sci USA*. (2014) 111:E2414–22. doi: 10.1073/pnas.1315605111
82. Sahin E, Colla S, Liesa M, Moslehi J, Muller FL, Guo M, et al. Telomere dysfunction induces metabolic and mitochondrial compromise. *Nature* (2011) 470:359–65. doi: 10.1038/nature09787
83. Hardie DG. AMPK: a target for drugs and natural products with effects on both diabetes and cancer. *Diabetes* (2013) 62:2164–72. doi: 10.2337/db13-0368
84. Li Y, Xu S, Mihaylova MM, Zheng B, Hou X, Jiang B, et al. AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. *Cell Metab*. (2011) 13:376–88. doi: 10.1016/j.cmet.2011.03.009
85. Toyama EQ, Herzig S, Courchet J, Lewis TL, Jr., Loson OC, Hellberg K, et al. Metabolism. AMP-activated protein kinase mediates mitochondrial fission in response to energy stress. *Science* (2016) 351:275–81. doi: 10.1126/science.aab4138
86. Sengupta S, Peterson TR, Sabatini DM. Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. *Mol Cell* (2010) 40:310–22. doi: 10.1016/j.molcel.2010.09.026
87. Duvel K, Yecies JL, Menon S, Raman P, Lipovsky AI, Souza AL, et al. Activation of a metabolic gene regulatory network downstream of mTOR complex 1. *Mol Cell* (2010) 39:171–83. doi: 10.1016/j.molcel.2010.06.022
88. Csibi A, Fendt SM, Li C, Poulogiannis G, Choo AY, Chapski DJ, et al. The mTORC1 pathway stimulates glutamine metabolism and cell proliferation by repressing SIRT4. *Cell* (2013) 153:840–54. doi: 10.1016/j.cell.2013.04.023
89. Ben-Sahra I, Hoxhaj G, Ricoult SJH, Asara JM, Manning BD. mTORC1 induces purine synthesis through control of the mitochondrial tetrahydrofolate cycle. *Science* (2016) 351:728–33. doi: 10.1126/science.aad0489
90. Porstmann T, Santos CR, Griffiths B, Cully M, Wu M, Leevers S, et al. SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth. *Cell Metab*. (2008) 8:224–36. doi: 10.1016/j.cmet.2008.07.007
91. Pylayeva-Gupta Y, Grabocka E, Bar-Sagi D. RAS oncogenes: weaving a tumorigenic web. *Nat Rev Cancer* (2011) 11:761–74. doi: 10.1038/nrc3106
92. Simanshu DK, Nissley DV, McCormick F. RAS proteins and their regulators in human disease. *Cell* (2017) 170:17–33. doi: 10.1016/j.cell.2017.06.009
93. Dias Carvalho P, Guimaraes CF, Cardoso AP, Mendonca S, Costa AM, Oliveira MJ, et al. KRAS oncogenic signaling extends beyond cancer cells to orchestrate the microenvironment. *Cancer Res*. (2018) 78:7–14. doi: 10.1158/0008-5472.CAN-17-2084
94. Karnoub AE, Weinberg RA. Ras oncogenes: split personalities. *Nat Rev Mol Cell Biol*. (2008) 9:517–31. doi: 10.1038/nrm2438
95. Ying H, Kimmelman AC, Lyssiotis CA, Hua S, Chu GC, Fletcher-Sananikone E, et al. Oncogenic Kras maintains pancreatic tumors through regulation of anabolic glucose metabolism. *Cell* (2012) 149:656–70. doi: 10.1016/j.cell.2012.01.058
96. Li X, Jiang Y, Meisenhelder J, Yang W, Hawke DH, Zheng Y, et al. Mitochondria-translocated PGK1 functions as a protein kinase to coordinate glycolysis and the TCA cycle in tumorigenesis. *Mol Cell* (2016) 61:705–19. doi: 10.1016/j.molcel.2016.02.009
97. Baracca A, Chiaradonna F, Sgarbi G, Solaini G, Alberghina L, Lenaz G. Mitochondrial Complex I decrease is responsible for bioenergetic dysfunction in K-ras transformed cells. *Biochim Biophys Acta* (2010) 1797:314–23. doi: 10.1016/j.bbabio.2009.11.006
98. Wang P, Song M, Zeng ZL, Zhu CF, Lu WH, Yang J, et al. Identification of NDUFAF1 in mediating K-Ras induced mitochondrial dysfunction by a proteomic screening approach. *Oncotarget* (2015) 6:3947–62. doi: 10.18632/oncotarget.2968
99. Rasola A, Sciacovelli M, Chiara F, Pantic B, Brusilow WS, Bernardi P. Activation of mitochondrial ERK protects cancer cells from death through inhibition of the permeability transition. *Proc Natl Acad Sci USA*. (2010) 107:726–31 doi: 10.1073/pnas.0912742107

100. Masgras I, Ciscato F, Brunati AM, Tibaldi E, Indraccolo S, Curtarello M, et al. Absence of neurofibromin induces an oncogenic metabolic switch via mitochondrial ERK-mediated phosphorylation of the chaperone TRAP1. *Cell Rep*. (2017) 18:659–72. doi: 10.1016/j.celrep.2016.12.056
101. Fan J, Kamphorst JJ, Mathew R, Chung MK, White E, Shlomi T, et al. Glutamine-driven oxidative phosphorylation is a major ATP source in transformed mammalian cells in both normoxia and hypoxia. *Mol Syst Biol*. (2013) 9:712. doi: 10.1038/msb.2013.65
102. Gaglio D, Metallo CM, Gameiro PA, Hiller K, Danna LS, Balestrieri C, et al. Oncogenic K-Ras decouples glucose and glutamine metabolism to support cancer cell growth. *Mol Syst Biol*. (2011) 7:523. doi: 10.1038/msb.2011.56
103. Yang D, Wang MT, Tang Y, Chen Y, Jiang H, Jones TT, et al. Impairment of mitochondrial respiration in mouse fibroblasts by oncogenic H-RAS(Q61L). *Cancer Biol Ther*. (2010) 9:122–33. doi: 10.4161/cbt.9.2.10379
104. Guo JY, Karsli-Uzunbas G, Mathew R, Aisner SC, Kamphorst JJ, Strohecker AM, et al. Autophagy suppresses progression of K-ras-induced lung tumors to oncocytomas and maintains lipid homeostasis. *Genes Dev*. (2013) 27:1447–61. doi: 10.1101/gad.219642.113
105. Weinberg F, Hamanaka R, Wheaton WW, Weinberg S, Joseph J, Lopez M, et al. Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. *Proc Natl Acad Sci USA*. (2010) 107:8788–93. doi: 10.1073/pnas.1003428107
106. Gaglio D, Soldati C, Vanoni M, Alberghina L, Chiaradonna F. Glutamine deprivation induces abortive s-phase rescued by deoxyribonucleotides in k-ras transformed fibroblasts. *PLoS ONE* (2009) 4:e4715. doi: 10.1371/journal.pone.0004715
107. Gao P, Tchernyshyov I, Chang TC, Lee YS, Kita K, Ochi T, et al. c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature* (2009) 458:762–5. doi: 10.1038/nature07823
108. Son J, Lyssiotis CA, Ying H, Wang X, Hua S, Ligorio M, et al. Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway. *Nature* (2013) 496:101–5. doi: 10.1038/nature12040
109. Kerr EM, Gaude E, Turrell FK, Frezza C, Martins CP. Mutant Kras copy number defines metabolic reprogramming and therapeutic susceptibilities. *Nature* (2016) 531:110–3. doi: 10.1038/nature16967
110. Astuti D, Latif F, Dallol A, Dahia PL, Douglas F, George E, et al. Gene mutations in the succinate dehydrogenase subunit SDHB cause susceptibility to familial pheochromocytoma and to familial paraganglioma. *Am J Hum Genet*. (2001) 69:49–54. doi: 10.1086/321282
111. Baysal BE, Ferrell RE, Willett-Brozick JE, Lawrence EC, Myssiorek D, Bosch A, et al. Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma. *Science* (2000) 287:848–51. doi: 10.1126/science.287.5454.848
112. Niemann S, Muller U. Mutations in SDHC cause autosomal dominant paraganglioma, type 3. *Nat Genet*. (2000) 26:268–70 doi: 10.1038/81551
113. Tomlinson IP, Alam NA, Rowan AJ, Barclay E, Jaeger EE, Kelsell D, et al. Germline mutations in FH predispose to dominantly inherited uterine fibroids, skin leiomyomata and papillary renal cell cancer. *Nat Genet*. (2002) 30:406–10. doi: 10.1038/ng849
114. Rustin P. Mitochondria, from cell death to proliferation. *Nat Genet*. (2002) 30:352–3. doi: 10.1038/ng0402-352
115. Dahia PL. Pheochromocytoma and paraganglioma pathogenesis: learning from genetic heterogeneity. *Nat Rev Cancer* (2014) 14:108–19. doi: 10.1038/nrc3648
116. Zhao T, Mu X, You Q. Succinate: an initiator in tumorigenesis and progression. *Oncotarget* (2017) 8:53819–28. doi: 10.18632/oncotarget.17734
117. van der Knaap JA, Verrijzer CP. Undercover: gene control by metabolites and metabolic enzymes. *Genes Dev*. (2016) 30:2345–69. doi: 10.1101/gad.289140.116
118. Kinnaird A, Zhao S, Wellen KE, Michelakis ED. Metabolic control of epigenetics in cancer. *Nat Rev Cancer* (2016) 16:694–707. doi: 10.1038/nrc.2016.82
119. Nowicki S, Gottlieb E. Oncometabolites: tailoring our genes. *FEBS J*. (2015) 282:2796–805. doi: 10.1111/febs.13295
120. Hoekstra AS, de Graaff MA, Briaire-de Bruijn IH, Ras C, Seifar RM, van Minderhout I, et al. Inactivation of SDH and FH cause loss of 5hmC and increased H3K9me3 in paraganglioma/pheochromocytoma and smooth muscle tumors. *Oncotarget* (2015) 6:38777–88. doi: 10.18632/oncotarget.6091
121. Ternette N, Yang M, Laroyia M, Kitagawa M, O'Flaherty L, Wolhulter K, et al. Inhibition of mitochondrial aconitase by succination in fumarate hydratase deficiency. *Cell Rep*. (2013) 3:689–700. doi: 10.1016/j.celrep.2013.02.013
122. Tyrakis PA, Yurkovich ME, Sciacovelli M, Papachristou EK, Bridges HR, Gaude E, et al. Fumarate hydratase loss causes combined respiratory chain defects. *Cell Rep*. (2017) 21:1036–47. doi: 10.1016/j.celrep.2017.09.092
123. Frezza C, Zheng L, Folger O, Rajagopalan KN, MacKenzie ED, Jerby L, et al. Haem oxygenase is synthetically lethal with the tumour suppressor fumarate hydratase. *Nature* (2011) 47:225–8. doi: 10.1038/nature10363
124. Nieto MA, Huang RY, Jackson RA, Thiery JP. Emt: 2016. *Cell* (2016) 166:21–45. doi: 10.1016/j.cell.2016.06.028
125. Sciacovelli M, Goncalves E, Johnson TI, Zecchini VR, da Costa AS, Gaude E, et al. Fumarate is an epigenetic modifier that elicits epithelial-to-mesenchymal transition. *Nature* (2016) 537:544–7. doi: 10.1038/nature19353
126. Adam J, Hatipoglu E, O'Flaherty L, Ternette N, Sahgal N, Lockstone H, et al. Renal cyst formation in Fh1-deficient mice is independent of the Hif/Phd pathway: roles for fumarate in KEAP1 succination and Nrf2 signaling. *Cancer Cell* (2011) 20:524–37. doi: 10.1016/j.ccr.2011.09.006
127. Sullivan LB, Martinez-Garcia E, Nguyen H, Mullen AR, Dufour E, Sudarshan S, et al. The proto-oncometabolite fumarate binds glutathione to amplify ROS-dependent signaling. *Mol Cell* (2013) 51:236–48. doi: 10.1016/j.molcel.2013.05.003
128. Gagne L, Boulay K, Topisirovic I, Huot ME, Mallette FA. Oncogenic activities of IDH1/2 mutations: from epigenetics to cellular signaling. *Trends Cell Biol*. (2017) 27:738–52. doi: 10.1016/j.tcb.2017.06.002
129. Xiao M, Yang H, Xu W, Ma S, Lin H, Zhu H, et al. Inhibition of alpha-KG-dependent histone and DNA demethylases by fumarate and succinate that are accumulated in mutations of FH and SDH tumor suppressors. *Genes Dev*. (2012) 26:1326–38. doi: 10.1101/gad.191056.112
130. Noushmehr H, Weisenberger DJ, Diefes K, Phillips HS, Pujara K, Berman BP, et al. Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell* (2010) 17:510–22. doi: 10.1016/j.ccr.2010.03.017
131. Dang L, Su SM. Isocitrate dehydrogenase mutation and (R)-2-hydroxyglutarate: from basic discovery to therapeutics development. *Annu Rev Biochem*. (2017) 86:305–31. doi: 10.1146/annurev-biochem-061516-044732
132. Rasola A, Neckers L, Picard D. Mitochondrial oxidative phosphorylation TRAP(1)ped in tumor cells. *Trends Cell Biol*. (2014) 24:455–63. doi: 10.1016/j.tcb.2014.03.005
133. Sciacovelli M, Guzzo G, Morello V, Frezza C, Zheng L, Nannini N, et al. The mitochondrial chaperone TRAP1 promotes neoplastic growth by inhibiting succinate dehydrogenase. *Cell Metab*. (2013) 17:988–99. doi: 10.1016/j.cmet.2013.04.019
134. Yoshida S, Tsutsumi S, Muhlebach G, Sourbier C, Lee MJ, Lee S, et al. Molecular chaperone TRAP1 regulates a metabolic switch between mitochondrial respiration and aerobic glycolysis. *Proc Natl Acad Sci USA*. (2013) 110:E1604–12. doi: 10.1073/pnas.1220659110
135. Ratner N, Miller SJ. A RASopathy gene commonly mutated in cancer: the neurofibromatosis type 1 tumour suppressor. *Nat Rev Cancer* (2015) 15:290–301. doi: 10.1038/nrc3911
136. Masgras I, Sanchez-Martin C, Colombo G, Rasola A. The chaperone TRAP1 as a modulator of the mitochondrial adaptations in cancer cells. *Front Oncol*. (2017) 7:58. doi: 10.3389/fonc.2017.00058
137. Holmstrom KM, Finkel T. Cellular mechanisms and physiological consequences of redox-dependent signalling. *Nat Rev Mol Cell Biol*. (2014) 15:411–21. doi: 10.1038/nrm3801
138. Shadel GS, Horvath TL. Mitochondrial ROS signaling in organismal homeostasis. *Cell* (2015) 163:560–9. doi: 10.1016/j.cell.2015.10.001
139. Sabharwal SS, Schumacker PT. Mitochondrial ROS in cancer: initiators, amplifiers or an Achilles' heel? *Nat Rev Cancer* (2014) 14:709–21. doi: 10.1038/nrc3803
140. Schumacker PT. Reactive oxygen species in cancer: a dance with the devil. *Cancer Cell* (2015) 27:156–7. doi: 10.1016/j.ccell.2015.01.007
141. Sies H, Berndt C, Jones DP. Oxidative stress. *Annu Rev Biochem*. (2017) 86:715–48. doi: 10.1146/annurev-biochem-061516-045037

142. Gorrini C, Harris IS, Mak TW. Modulation of oxidative stress as an anticancer strategy. *Nat Rev Drug Discov*. (2013) 12:931–47 doi: 10.1038/nrd4002
143. Sullivan LB, Chandel NS. Mitochondrial reactive oxygen species and cancer. *Cancer Metab*. (2014) 2:17. doi: 10.1186/2049-3002-2-17
144. Reczek CR, Chandel NS. ROS-dependent signal transduction. *Curr Opin Cell Biol*. (2015) 33:8–13. doi: 10.1016/j.ceb.2014.09.010
145. Saunier E, Benelli C, Bortoli S. The pyruvate dehydrogenase complex in cancer: An old metabolic gatekeeper regulated by new pathways and pharmacological agents. *Int J Cancer* (2016) 138:809–17. doi: 10.1002/ijc.29564
146. Finley LW, Haas W, Desquiret-Dumas V, Wallace DC, Procaccio V, Gygi SP, et al. Succinate dehydrogenase is a direct target of sirtuin 3 deacetylase activity. *PLoS ONE* (2011) 6:e23295. doi: 10.1371/journal.pone.0023295
147. Xie H, Simon MC. Oxygen availability and metabolic reprogramming in cancer. *J Biol Chem*. (2017) 292:16825–32. doi: 10.1074/jbc.R117.799973
148. Fukuda R, Zhang H, Kim JW, Shimoda L, Dang CV, Semenza GL. HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells. *Cell* (2007) 129:111–22. doi: 10.1016/j.cell.2007.01.047
149. Chourasia AH, Boland ML, Macleod KF. Mitophagy and cancer. *Cancer Metab*. (2015) 3:4. doi: 10.1186/s40170-015-0130-8
150. Matsuda S, Nakanishi A, Minami A, Wada Y, Kitagishi Y. Functions and characteristics of PINK1 and Parkin in cancer. *Front Biosci (Landmark Ed)* (2015) 20:491–501 doi: 10.2741/4321
151. Hayes JD, Dinkova-Kostova AT. The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. *Trends Biochem Sci*. (2014) 39:199–218. doi: 10.1016/j.tibs.2014.02.002
152. Menegon S, Columbano A, Giordano S. The dual roles of NRF2 in cancer. *Trends Mol Med*. (2016) 22:578–93. doi: 10.1016/j.molmed.2016.05.002
153. Jaramillo MC, Zhang DD. The emerging role of the Nrf2-Keap1 signaling pathway in cancer. *Genes Dev*. (2013) 27:2179–91. doi: 10.1101/gad.225680.113
154. Kowalik MA, Guzzo G, Morandi A, Perra A, Menegon S, Masgras I, et al. Metabolic reprogramming identifies the most aggressive lesions at early phases of hepatic carcinogenesis. *Oncotarget* (2016) 7:32375–93. doi: 10.18632/oncotarget.8632
155. Guzzo G, Sciacovelli M, Bernardi P, Rasola A. Inhibition of succinate dehydrogenase by the mitochondrial chaperone TRAP1 has anti-oxidant and anti-apoptotic effects on tumor cells. *Oncotarget* (2014) 5:11897–908. doi: 10.18632/oncotarget.2472
156. Clapham DE. Calcium signaling. *Cell* (2007) 131:1047–58. doi: 10.1016/j.cell.2007.11.028
157. Filadi R, Pozzan T. Generation and functions of second messengers microdomains. *Cell Calcium* (2015) 58:405–14. doi: 10.1016/j.ceca.2015.03.007
158. Rizzuto R, De Stefani D, Raffaello A, Mammucari C. Mitochondria as sensors and regulators of calcium signalling. *Nat Rev Mol Cell Biol*. (2012) 13:566–78. doi: 10.1038/nrm3412
159. Akl H, Bultynck G. Altered Ca(2+) signaling in cancer cells: proto-oncogenes and tumor suppressors targeting IP3 receptors. *Biochim Biophys Acta* (2013) 1835:180–93. doi: 10.1016/j.bbcan.2012.12.001
160. Bittremieux M, Parys JB, Pinton P, Bultynck G. ER functions of oncogenes and tumor suppressors: Modulators of intracellular Ca(2+) signaling. *Biochim Biophys Acta* (2016) 1863:1364–78. doi: 10.1016/j.bbamcr.2016.01.002
161. Bernardi P, Rasola A, Forte M, Lippe G. The mitochondrial permeability transition pore: channel formation by F-ATP synthase, integration in signal transduction, and role in pathophysiology. *Physiol Rev*. (2015) 95:1111–55. doi: 10.1152/physrev.00001.2015
162. Rasola A, Bernardi P. Mitochondrial permeability transition in Ca(2+)-dependent apoptosis and necrosis. *Cell Calcium* (2011) 50:222–33. doi: 10.1016/j.ceca.2011.04.007
163. Rasola A, Bernardi P. The mitochondrial permeability transition pore and its adaptive responses in tumor cells. *Cell Calcium* (2014) 56:437–45. doi: 10.1016/j.ceca.2014.10.003
164. Cardenas C, Muller M, McNeal A, Lovy A, Jana F, Bustos G, et al. Selective vulnerability of cancer cells by inhibition of Ca(2+) transfer from endoplasmic reticulum to mitochondria. *Cell Rep*. (2016) 14:2313–24. doi: 10.1016/j.celrep.2016.02.030
165. Tarasov AI, Griffiths EJ, Rutter GA. Regulation of ATP production by mitochondrial Ca(2+). *Cell Calcium* (2012) 52:28–35. doi: 10.1016/j.ceca.2012.03.003
166. Hurst S, Hoek J, Sheu SS. Mitochondrial Ca(2+) and regulation of the permeability transition pore. *J Bioenerg Biomembr*. (2017) 49:27–47. doi: 10.1007/s10863-016-9672-x
167. Contreras L, Gomez-Puertas P, Iijima M, Kobayashi K, Saheki T, Satrustegui J. Ca2+ Activation kinetics of the two aspartate-glutamate mitochondrial carriers, aralar and citrin: role in the heart malate-aspartate NADH shuttle. *J Biol Chem*. (2007) 282:7098–106. doi: 10.1074/jbc.M610491200
168. Ren T, Zhang H, Wang J, Zhu J, Jin M, Wu Y, et al. MCU-dependent mitochondrial Ca(2+) inhibits NAD(+)/SIRT3/SOD2 pathway to promote ROS production and metastasis of HCC cells. *Oncogene* (2017) 36:5897–909. doi: 10.1038/onc.2017.167
169. Tosatto A, Sommaggio R, Kummerow C, Bentham RB, Blacker TS, Berecz T, et al. The mitochondrial calcium uniporter regulates breast cancer progression via HIF-1alpha. *EMBO Mol Med*. (2016) 8:569–85. doi: 10.15252/emmm.201606255
170. Hofer A, Wenz T. Post-translational modification of mitochondria as a novel mode of regulation. *Exp Gerontol*. (2014) 56:202–20. doi: 10.1016/j.exger.2014.03.006
171. Stram AR, Payne RM. Post-translational modifications in mitochondria: protein signaling in the powerhouse. *Cell Mol Life Sci*. (2016) 73:4063–73. doi: 10.1007/s00018-016-2280-4
172. Hosp F, Lassowskat I, Santoro V, De Vleesschauwer D, Fliegner D, Redestig H, et al. Lysine acetylation in mitochondria: From inventory to function. *Mitochondrion* (2017) 33:58–71. doi: 10.1016/j.mito.2016.07.012
173. Baeza J, Smallegan MJ, Denu JM. Mechanisms and dynamics of protein acetylation in mitochondria. *Trends Biochem Sci*. (2016) 41:231–44. doi: 10.1016/j.tibs.2015.12.006
174. Scott I, Wang L, Wu K, Thapa D, Sack MN. GCN5L1/BLOS1 Links Acetylation, Organelle Remodeling, and Metabolism. *Trends Cell Biol*. (2018) 28:346–55. doi: 10.1016/j.tcb.2018.01.007
175. van de Ven RAH, Santos D, Haigis MC. Mitochondrial Sirtuins and Molecular Mechanisms of Aging. *Trends Mol Med*. (2017) 23:320–31. doi: 10.1016/j.molmed.2017.02.005
176. Houtkooper RH, Pirinen E, Auwerx J. Sirtuins as regulators of metabolism and healthspan. *Nat Rev Mol Cell Biol*. (2012) 13:225–38. doi: 10.1038/nrm3293
177. Mei Z, Zhang X, Yi J, Huang J, He J, Tao Y. Sirtuins in metabolism, DNA repair and cancer. *J Exp Clin Cancer Res*. (2016) 35:182. doi: 10.1186/s13046-016-0461-5
178. Chalkiadaki A, Guarente L. The multifaceted functions of sirtuins in cancer. *Nat Rev Cancer* (2015) 15:608–24. doi: 10.1038/nrc3985
179. Song BJ, Akbar M, Abdelmegeed MA, Byun K, Lee B, Yoon SK, et al. Mitochondrial dysfunction and tissue injury by alcohol, high fat, nonalcoholic substances and pathological conditions through post-translational protein modifications. *Redox Biol*. (2014) 3:109–23. doi: 10.1016/j.redox.2014.10.004
180. Acin-Perez R, Gatti DL, Bai Y, Manfredi G. Protein phosphorylation and prevention of cytochrome oxidase inhibition by ATP: coupled mechanisms of energy metabolism regulation. *Cell Metab*. (2011) 13:712–9. doi: 10.1016/j.cmet.2011.03.024
181. Nesci S, Trombetti F, Ventrella V, Pagliarani A. Post-translational modifications of the mitochondrial F1FO-ATPase. *Biochim Biophys Acta* (2017) 1861:2902–12. doi: 10.1016/j.bbagen.2017.08.007
182. Hebert-Chatelain E. Src kinases are important regulators of mitochondrial functions. *Int J Biochem Cell Biol*. (2013) 45:90–8. doi: 10.1016/j.biocel.2012.08.014
183. Acin-Perez R, Carrascoso I, Baixauli F, Roche-Molina M, Latorre-Pellicer A, Fernandez-Silva P, et al. ROS-triggered phosphorylation of complex II by Fgr kinase regulates cellular adaptation to fuel use. *Cell Metab*. (2014) 19:1020–33. doi: 10.1016/j.cmet.2014.04.015
184. Chiara F, Rasola A. GSK-3 and mitochondria in cancer cells. *Front Oncol*. (2013) 3:16. doi: 10.3389/fonc.2013.00016

185. Schrepfer E, Scorrano L. Mitofusins, from mitochondria to metabolism. *Mol Cell* (2016) 61:683–94. doi: 10.1016/j.molcel.2016.02.022
186. Senft D, Ronai ZA. Regulators of mitochondrial dynamics in cancer. *Curr Opin Cell Biol* (2016) 39:43–52. doi: 10.1016/j.ceb.2016.02.001
187. Trotta AP, Chipuk JE. Mitochondrial dynamics as regulators of cancer biology. *Cell Mol Life Sci*. (2017) 74:1999–2017. doi: 10.1007/s00018-016-2451-3
188. Chen H, Chan DC. Mitochondrial dynamics in regulating the unique phenotypes of cancer and stem cells. *Cell Metab*. (2017) 26:39–48. doi: 10.1016/j.cmet.2017.05.016
189. Simula L, Nazio F, Campello S. The mitochondrial dynamics in cancer and immune-surveillance. *Semin Cancer Biol*. (2017) 47:29–42. doi: 10.1016/j.semcancer.2017.06.007
190. Ashrafi G, Schwarz TL. The pathways of mitophagy for quality control and clearance of mitochondria. *Cell Death Differ*. (2013) 20:31–42. doi: 10.1038/cdd.2012.81
191. Drake LE, Springer MZ, Poole LP, Kim CJ, Macleod KF. Expanding perspectives on the significance of mitophagy in cancer. *Semin Cancer Biol*. (2017) 47:110–24. doi: 10.1016/j.semcancer.2017.04.008
192. White E, Mehnert JM, Chan CS. Autophagy, metabolism, and cancer. *Clin Cancer Res*. (2015) 21:5037–46. doi: 10.1158/1078-0432.CCR-15-0490
193. Hotamisligil GS. Foundations of immunometabolism and implications for metabolic health and disease. *Immunity* (2017) 47:406–20. doi: 10.1016/j.immuni.2017.08.009
194. Morandi A, Giannoni E, Chiarugi P. Nutrient exploitation within the tumor-stroma metabolic crosstalk. *Trends Cancer* (2016) 2:736–46. doi: 10.1016/j.trecan.2016.11.001
195. Przybyla L, Muncie JM, Weaver VM. Mechanical control of epithelial-to-mesenchymal transitions in development and cancer. *Annu Rev Cell Dev Biol*. (2016) 32:527–54. doi: 10.1146/annurev-cellbio-111315-125150
196. DelNero P, Hopkins BD, Cantley LC, Fischbach C. Cancer metabolism gets physical. *Sci Transl Med*. (2018) 10:eaaq1011. doi: 10.1126/scitranslmed.aaq1011
197. Alberghina L, Gaglio D. Redox control of glutamine utilization in cancer. *Cell Death Dis*. (2014) 5:e1561. doi: 10.1038/cddis.2014.513
198. Pavlova NN, Thompson CB. The Emerging Hallmarks of Cancer Metabolism. *Cell Metab* (2016) 23:27–47. doi: 10.1016/j.cmet.2015.12.006
199. Carracedo A, Cantley LC, Pandolfi PP. Cancer metabolism: fatty acid oxidation in the limelight. *Nat Rev Cancer* (2013) 13:227–32. doi: 10.1038/nrc3483
200. Wise DR, Ward PS, Shay JE, Cross JR, Gruber JJ, Sachdeva UM, et al. Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of alpha-ketoglutarate to citrate to support cell growth and viability. *Proc Natl Acad Sci USA*. (2011) 108:19611–6. doi: 10.1073/pnas.1117773108
201. Jiang L, Shestov AA, Swain P, Yang C, Parker SJ, Wang QA, et al. Reductive carboxylation supports redox homeostasis during anchorage-independent growth. *Nature* (2016) 532:255–8. doi: 10.1038/nature17393
202. Chen J, Guccini I, Mitri DD, Brina D, Revandkar A, Sarti M, et al. Compartmentalized activities of the pyruvate dehydrogenase complex sustain lipogenesis in prostate cancer. *Nat Genet*. (2018) 50:219–28. doi: 10.1038/s41588-017-0026-3
203. Linehan WM, Rouault TA. Molecular pathways: fumarate hydratase-deficient kidney cancer–targeting the Warburg effect in cancer. *Clin Cancer Res*. (2013) 19:3345–52. doi: 10.1158/1078-0432.CCR-13-0304
204. Cardaci S, Zheng L, MacKay G, van den Broek NJ, MacKenzie ED, Nixon C, et al. Pyruvate carboxylation enables growth of SDH-deficient cells by supporting aspartate biosynthesis. *Nat Cell Biol*. (2015) 17:1317–26. doi: 10.1038/ncb3233
205. Lussey-Lepoutre C, Hollinshead KE, Ludwig C, Menara M, Morin A, Castro-Vega LJ, et al. Loss of succinate dehydrogenase activity results in dependency on pyruvate carboxylation for cellular anabolism. *Nat Commun*. (2015) 6:8784. doi: 10.1038/ncomms9784
206. Peiris-Pages M, Martinez-Outschoorn UE, Pestell RG, Sotgia F, Lisanti MP. Cancer stem cell metabolism. *Breast Cancer Res* (2016) 18:55. doi: 10.1186/s13058-016-0712-6
207. Lyssiotis CA, Kimmelman AC. Metabolic interactions in the tumor microenvironment. *Trends Cell Biol*. (2017) 27:863–75. doi: 10.1016/j.tcb.2017.06.003
208. Ye H, Adane B, Khan N, Sullivan T, Minhajuddin M, Gasparetto M, et al. Leukemic stem cells evade chemotherapy by metabolic adaptation to an adipose tissue niche. *Cell Stem Cell* (2016) 19:23–37. doi: 10.1016/j.stem.2016.06.001
209. Berridge MJ. Calcium hypothesis of Alzheimer's disease. *Pflugers Arch* (2010) 459:441-9. doi: 10.1007/s00424-009-0736-1
210. Curtarello M, Zulato E, Nardo G, Valtorta S, Guzzo G, Rossi E, et al. VEGF-targeted therapy stably modulates the glycolytic phenotype of tumor cells. *Cancer Res*. (2015) 75:120–33. doi: 10.1158/0008-5472.CAN-13-2037

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 09 July 2018
doi: 10.3389/fonc.2018.00254

LonP1 Differently Modulates Mitochondrial Function and Bioenergetics of Primary Versus Metastatic Colon Cancer Cells

*Lara Gibellini[1], Lorena Losi[2], Sara De Biasi[2], Milena Nasi[3], Domenico Lo Tartaro[1], Simone Pecorini[3], Simone Patergnani[4], Paolo Pinton[4], Anna De Gaetano[3], Gianluca Carnevale[3], Alessandra Pisciotta[3], Francesco Mariani[5], Luca Roncucci[5], Anna Iannone[5], Andrea Cossarizza[1] and Marcello Pinti[2]**

[1] Department of Medical and Surgical Sciences for Children and Adults, University of Modena and Reggio Emilia, Modena, Italy, [2] Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy, [3] Department of Surgery, Medicine, Dentistry and Morphological Sciences, University of Modena and Reggio Emilia, Modena, Italy, [4] Department of Morphology, Surgery and Experimental Medicine, Section of Pathology, Oncology and Experimental Biology and LTTA Center, University of Ferrara, Ferrara, Italy, [5] Department of Diagnostic, Clinical Medicine and Public Health, University of Modena and Reggio Emilia, Modena, Italy

OPEN ACCESS

Edited by:
Andrea Rasola, Università degli Studi di Padova, Italy

Reviewed by:
Paolo E. Porporato, Università degli Studi di Torino, Italy
Carlo Pucillo, Università degli Studi di Udine, Italy

***Correspondence:**
Marcello Pinti
marcello.pinti@unimore.it

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *01 February 2018*
Accepted: *21 June 2018*
Published: *09 July 2018*

Citation:
Gibellini L, Losi L, De Biasi S, Nasi M, Lo Tartaro D, Pecorini S, Patergnani S, Pinton P, De Gaetano A, Carnevale G, Pisciotta A, Mariani F, Roncucci L, Iannone A, Cossarizza A and Pinti M (2018) LonP1 Differently Modulates Mitochondrial Function and Bioenergetics of Primary Versus Metastatic Colon Cancer Cells. Front. Oncol. 8:254. doi: 10.3389/fonc.2018.00254

Mitochondrial Lon protease (LonP1) is a multi-function enzyme that regulates mitochondrial functions in several human malignancies, including colorectal cancer (CRC). The mechanism(s) by which LonP1 contributes to colorectal carcinogenesis is not fully understood. We found that silencing LonP1 leads to severe mitochondrial impairment and apoptosis in colon cancer cells. Here, we investigate the role of LonP1 in mitochondrial functions, metabolism, and epithelial–mesenchymal transition (EMT) in colon tumor cells and in metastasis. LonP1 was almost absent in normal mucosa, gradually increased from aberrant crypt foci to adenoma, and was most abundant in CRC. Moreover, LonP1 was preferentially upregulated in colorectal samples with mutated p53 or nuclear β-catenin, and its overexpression led to increased levels of β-catenin and decreased levels of E-cadherin, key proteins in EMT, *in vitro*. LonP1 upregulation also induced opposite changes in oxidative phosphorylation, glycolysis, and pentose pathway in SW480 primary colon tumor cells when compared to SW620 metastatic colon cancer cells. In conclusion, basal LonP1 expression is essential for normal mitochondrial function, and increased LonP1 levels in SW480 and SW620 cells induce a metabolic shift toward glycolysis, leading to EMT.

Keywords: LonP1, mitochondria, bioenergetics, beta-catenin, colorectal cancer, protease

INTRODUCTION

LonP1 (also known as Lon or LonP) is one of the main proteases patrolling the mitochondrial matrix. Lon is a multi-function enzyme, exerting both proteolytic and chaperone activities, and also binds mitochondrial DNA and RNA [reviewed in Ref. (1)]. Main targets of LonP1 proteolytic activity are: (i) folded proteins, including 5-aminolevulinic acid synthase, steroidogenic acute regulatory protein, mitochondrial transcription factor A, and cytochrome c oxidase 4 isoform 1; (ii) misfolded proteins, including glutaminase C; (iii) oxidized proteins, including aconitase and cystathionine β-synthase

(2–8). Conversely, targets of LonP1 chaperone activity are still not known. Initial studies demonstrated that LonP1 was involved in mitochondrial maintenance and mitochondrial quality control during aging (9, 10). More recent evidence suggests that LonP1 is responsible for additional functions critical to tumor progression, including metabolic adaptation to hypoxia, protection against senescence, and resistance to apoptosis and oxidative stress (11–14). Recent data from our group demonstrated that LonP1 is upregulated in colorectal tumors, and that its downregulation or inhibition leads to severe mitochondrial dysfunction and to apoptosis (15, 16).

Colorectal cancer (CRC) is the third most common cancer worldwide (17). Despite several advances have been made in the diagnosis and treatment of CRC over the past decades, the overall prognosis still remains poor, and tumor metastasis represent a major obstacle to successful treatment (18). The epithelial–mesenchymal transition (EMT) is a biologic process that enables a polarized epithelial cell to assume a mesenchymal cell phenotype through biochemical changes and a number of distinct molecular processes (19). Several intracellular signaling pathways contribute to EMT, and involve ERK, MAPK, PI3K, Akt, Smads, β-catenin (β-ctn), Ras, c-fos, and among others (19). The Wnt/β-ctn signaling pathway has a crucial role in the negative regulation of E-cadherin, and in the development of EMT and CRC metastasis (20). In normal conditions, and in the absence of activated Wnt signals, β-ctn is phosphorylated by APC/Axin/GSK-3β complex and then degraded by the proteasome. When Wnt ligands activate Frizzled and LPR receptors, β-ctn is no longer phosphorylated and translocates into the nucleus where it binds to transcription factors belonging to the family of T-cell factor (TCF) and lymphoid enhancer-binding protein, and then it activates transcription (21). In CRC, the vast majority of tumors have mutations in the key regulatory factors of the Wnt/β-ctn pathway and up to 80% present nuclear accumulation of β-ctn (20).

Starting from our previous observations, we aimed at investigating the precise role of LonP1 in colon primary tumor and metastasis. In this study, we show that elevated expression of LonP1 in CRC tissues is associated with nuclear localization of β-ctn, and that overexpression of LonP1, *in vitro*, differently affects β-ctn expression in SW480 and SW620 colon cancer cells. We also show that LonP1 overexpression differently affects mitochondrial functions and bioenergetics in SW480 and SW620 cells.

MATERIALS AND METHODS

Cell Culture

Four cancer cell lines were used for this study: I407 intestinal epithelial cells, SW480 and RKO colon carcinoma cells, and SW620 metastatic colon cancer cells. SW480 cells were cultured in DMEM high glucose supplemented with 10% fetal bovine serum (FBS) and gentamycin. I407, RKO, and SW620 cells were cultured in RPMI Glutamax supplemented with 10% FBS and gentamycin. Cells were maintained in 5% CO_2 atmosphere at 37°C. Culture media and reagents were from ThermoFisher Scientific (Eugene, OR, USA).

Human Colorectal Tissues

We have studied a total of 45 patients who underwent surgical removal of CRC. Samples used for immunohistochemistry were formalin-fixed paraffin-embedded (FFPE) specimens, the other samples had been freshly frozen with a passage in liquid nitrogen, and then stored at −80°C until use. Tissues were obtained from the Department of Diagnostic and Clinical Medicine, and Public Health, University of Modena and Reggio Emilia, through an institutional review board-approved protocol. Demographic and clinical characteristics of patients are reported in **Tables 1** and **2**.

Retroviral Transduction

The pMSCV-Puro empty vector and the pMSCV containing the cDNA encoding for Lon protease (hereafter referred to as pLonP1) were used to transiently transfect amphotrophic Phoenix cell line (15). Cells infected with the empty vector will be indicated as pMSCV cells. Retroviral supernatants were used to stably transfect I407, RKO, SW480, and SW620 cells, and stable transfectants were selected by using 3–4 μg/ml puromycin (depending on the cell line), and then maintained in cell medium supplemented with 2 μg/ml puromycin.

RNA Interference

Cells were reverse transfected by using RNAiMAx (Life Technologies Corporation) and 10 nM s17901 small interfering RNAs (Life Technologies Corporation) against LonP1 mRNA. Then, cells were incubated for 72 h, trypsinized, and lysated by using RIPA buffer.

TABLE 1 | Demographic characteristics and tumor stage in samples obtained from NM, ACF, Ad, or CRC.

Patient	Stage	Age	Sex
1	NM	84	M
2	NM	85	M
3	NM	78	M
4	NM	86	M
5	NM	84	M
6	NM	46	M
7	NM	52	F
8	NM	82	M
9	NM	46	F
10	NM	86	M
11	NM	43	M
12	NM	82	M
13	ACF	38	F
14	ACF	43	M
15	ACF	46	F
16	ACF	38	F
17	ACF	43	M
18	ACF	43	M
16	Ad	38	F
17	Ad	43	M
18	Ad	46	F
19	Ad	46	F
20	Ad	43	M
21	Ad	43	M
22	CRC	85	M
23	CRC	86	M
24	CRC	46	F

M, male; F, female; NM, normal mucosa; ACF, aberrant crypt foci; Ad, adenoma; CRC, colorectal cancer.

TABLE 2 | Demographic characteristics, tumor stage, Ki-67, β-catenin (β-ctn), E-cadherin (E-cad), p53, LonP1 expression in 21 colorectal cancer samples.

Patient	Age	Sex	Stage	Site	Ki-67 (%)	β-ctn	E-cad	p53	Lon IHC	LonP1 Wb
1	73	M	II	Ascending	90	N	P	+	>75%	0.92
2	75	M	II	Rectum	60	N	P	+	>75%	4.06
3	55	F	I	Rectum	60	N	P	+	>75%	3.45
4	61	F	III	Rectum	50	Me	P	+	>75%	2.10
5	59	F	II	Sigmoid	40	N	P	+	>75%	76.70
6	61	F	III	Rectum	60	Me	P	+	>75%	4.94
7	83	F	III	Rectum	60	Me	P	–	>75%	1.37
8	66	F	I	Rectum	80	N	P	+	>75%	35.30
9	81	F	III	Ascending	70	Me	P	+	30–75%	48.85
10	63	M	IV	Ascending	40	Me	P	+	<30%	0.27
11	72	M	III	Ascending	60	Me	P	–	30–75%	2.55
12	88	M	I	S-R	70	Me	P	–	<30%	0.64
13	80	M	IV	S-R	40	N	P	–	<30%	3.60
14	71	F	II	S-R	40	N	P	–	30–75%	2.53
15	71	M	IV	Rectum	80	N	P	–	30–75%	1.17
16	47	M	IV	S-R	80	N	P	+	30–75%	7.47
17	62	F	IV	Ascending	50	Me	P	–	<30%	0.16
18	73	F	I	Rectum	70	N	P	+	30–75%	3.56
19	60	F	IV	Rectum	40	Me	P	+	<30%	0.10
20	56	M	III	Rectum	60	N	P	–	<30%	0.29
21	75	F	I	Rectum	70	Me	P	–	30–75%	1.04

M, male; F, female; Me, membrane; N, nuclear; P, present.

Immunohistochemical Analyses

Tumor specimens were taken from patients who underwent surgical resection of the large bowel in the period 2010–2015. All slides were blindly reviewed by the pathologist (LL). For each case, a representative paraffin-embedded block containing tumor tissue and normal mucosa, as internal control, was sectioned at 4 μm. Immunoperoxidase staining was run with the Benchmark XT Automatic Staining System (Ventana Roche) with diaminobenzidine as chromogen and using the View DAB Detection Kit (Roche). At the end of the reaction, slides were counterstained with hematoxylin. The following antibodies were used: mouse monoclonal antibodies β-ctn (Roche, Basilea, Switzerland), p53 (Roche), and E-cadherin (Dako, Santa Clara, CA, USA) available as pre-diluted commercially preparations; mouse monoclonal Ki-67 antibody (MIB-1, Dako, used at 1:100 dilution), and rabbit polyclonal LonP1 antibody (Primm, Milan, Italy, used at a 1:500 dilution). According to previous reports, expression of β-ctn and E-cadherin in normal colon epithelium resulted to be restricted to cell membrane. Altered expression of β-ctn was contemplated when 10% of tumor cells or greater showed nuclear or cytoplasmic immunoreactivity. Loss of membrane expression of E-cadherin was considered in cases exhibiting either no immunoreactivity or 10% of tumor cells or less with positive membranous staining. Detection of proliferative activity was carried out using an anti-Ki-67 antibody, and basal cells of the normal colonic crypts were used as internal positive control. Ki-67 labeling index was determined by counting the number of positive nuclei for 1,000 neoplastic cells in 10 consecutive fields chosen randomly in non-necrotic areas of the tumor.

Immunoblotting

Total protein lysate was obtained from cell lines by using RIPA buffer and from FFPE tissue as previously described (22). Protein concentration was determined by Bradford assay. Proteins were then separated in 4–12% or 12% Bolt Bis-Tris precast gels (Thermo Fisher Corporation) and transferred onto nitrocellulose membranes. Protein transfer was performed by using methanol transfer buffer or by using Trans-Blot Turbo cassettes (Bio-Rad Laboratories) with Trans-Blot Turbo blotting system (Bio-Rad Laboratories). Rabbit polyclonal anti-LonP1 was a custom antibody from Primm (Milan, Italy). Rabbit polyclonal anti-β-actin and rabbit polyclonal anti-E-cadherin were from Abcam (Cambridge, UK). Rabbit polyclonal to β-ctn, to lactate dehydrogenase A (LDHA), to glucose 6-phosphate dehydrogenase (G6PD), to N-cadherin, to Akt, to phospho-Akt (Ser473), to GSK-3β, to phospho-GSK-3β (Ser9), and to Twist were from Cell Signaling Technology (Danvers, MA, USA). Images were acquired by using ChemiDoc MP (Bio-Rad Laboratories) and Image Lab version 5.2.1 was used to perform densitometric analysis.

Immunofluorescence

Cells were grown on coverslips and fixed for 15 min in PBS containing 4% paraformaldehyde. Samples were then washed three times with PBS, incubated in PBS containing 0.1% Triton X-100 for 1 h at room temperature, and blocked by using PBS containing 3% bovine serum albumin for 1 h. Samples were then incubated with PBS containing primary antibody and secondary antibodies for 60 and 30 min, respectively. The following antibodies were used: rabbit polyclonal anti-β-ctn (Cell Signaling Technology) and anti-rabbit Alexa Fluor 647. Images were acquired by using a Nikon A1 confocal laser scanning microscope (Nikon, Tokyo, Japan).

Transmission Electron Microscopy

Cell pellets were fixed in 2.5% glutaraldehyde in Sorensen's Phosphate Buffer 0.1 M pH 7.4 (PB) for 1 h and post-fixed in

1% OsO_4 in PB 0.1 M for 1 h, as previously described (23). Then, ultrathin sections were cut from Durcupan embedded samples, collected on nickel grids, stained with uranyl acetate and lead-citrate, and then analyzed by using a Zeiss EM 109 Transmission Electron Microscope (Zeiss AG, Jena, Germany).

Oxygen Consumption Rate (OCR)

The rate of oxygen consumption was assayed with the XF96 Extracellular Flux Analyzer (Seahorse Biosciences—Agilent Technologies, Santa Clara, CA USA). Cells were plated 2 days before the experiment, and experiments were performed on a confluent monolayer. The number of cells that were plated was: 5×10^4/well for SW620-pMSCV and SW620-pLonP1, and 4×10^4/well for SW480-pMSCV and SW480-pLonP1.

Cytofluorimetric Analyses

Flow cytometry was used to determine mitochondrial mass, mitochondrial membrane potential (MMP), mitochondrial reactive oxygen species (ROS), and glucose transporter (GLUT)-1 expression. For mitochondrial mass analysis, cells were incubated with MitoTracker Green FM (MTG, 200 nM, Thermo Fisher Corporation) for 30 min at 37°C. For MMP analysis, cells were incubated with tetramethylrhodamine, methyl ester (TMRM, 200 nM, Thermo Fisher Corporation) for 10 min at 37°C. Carbonyl cyanide m-chlorophenyl hydrazine (CCCP, 1 and 10 μM, Sigma Aldrich) was used to induce mitochondrial membrane depolarization. Mitochondrial superoxide production was assessed using MitoSox Red Mitochondrial Superoxide Indicator (mtSOX, 5 μM, ThermoFischer Corporation). GLUT-1 expression was assessed by staining cells with anti-GLUT-1 Alexa Fluor 488 (Abcam). Data were collected on an Attune NxT (Thermo Fisher Corporation) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

Statistical Analysis

Quantitative variables were compared with non-parametric Mann–Whitney test. Statistical analyses were performed using GraphPad 5.0 (Prism, La Jolla, CA, USA). Error bars represent SD. A p value <0.05 was considered significant.

RESULTS

LonP1 Is Upregulated in CRC and Is Associated With Mutated p53 or Nuclear β-ctn

We previously demonstrated that LonP1 silencing is associated with severe mitochondrial dysfunction and apoptosis susceptibility in colon cancer cell lines (22). Herein, we quantified the expression of LonP1 during colon cancer progression in fresh frozen tissues of ACF, adenoma (Ad), and CRC from a total of 24 patients, whose demographic and clinical characteristics are reported in **Table 1**. We found that the LonP1 was almost absent in normal mucosa, gradually increased from samples of ACF to Ad, and was most abundant in samples of CRC (**Figures 1A,B**). In addition, we quantified LonP1 levels in FFPE samples from 21 patients affected by CRC, whose demographic and clinical characteristics are reported in **Table 2**. Despite inter-individuals variations, LonP1 was upregulated in CRC samples compared with the normal mucosa counterpart (**Figures 2A–D**).

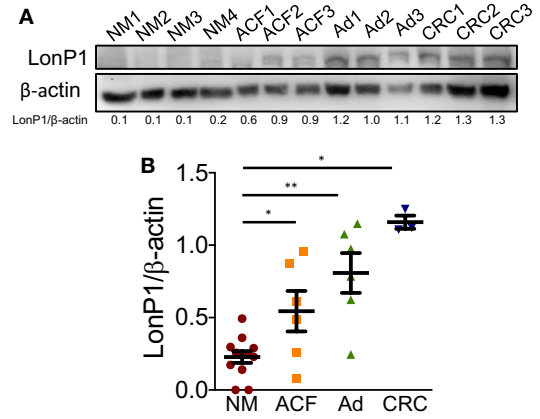


FIGURE 1 | LonP1 expression during colon cancer progression. **(A)** Representative Western blot analysis of LonP1 expression in freshly frozen tissue samples from normal mucosa (NM), aberrant crypt foci (ACF), colonic adenoma (Ad), and colorectal cancer (CRC). **(B)** Relative protein level of LonP1 in NM, ACF, Ad, and CRC. Data are reported as mean ± SD; $^*P < 0.05$ and $^{**}P < 0.01$.

In the same FFPE samples, we explored the expression levels of EMT-related proteins together with mutated p53 and Ki-67, and their association with LonP1. Representative images of immunohistochemical staining are reported in **Figure 3A**. We found that LonP1 was preferentially upregulated in colorectal samples with mutated p53 or nuclear β-ctn (**Figure 3B**). No differences were observed regarding Ki-67 levels.

LonP1 Modulation Is Associated With Changes in β-ctn Levels

To investigate the link between LonP1 and EMT in colon, we took advantage of four different cell lines, I407, RKO, SW480, and SW620 cells. I407 are intestinal epithelial immortalized cells. RKO and SW480 are colon carcinoma cells, whereas SW620 are metastatic colon cancer cells. Moreover, SW480 and SW620 represent a primary adenocarcinoma tumor and a lymph node metastasis from the same patient, respectively. We first established stable LonP1 overexpression in SW480 cells, and quantified the levels of several EMT-related proteins, including β-ctn, E-cadherin, and N-cadherin. Forced LonP1 expression led to increased β-ctn levels, reduced E-cadherin, and increased N-cadherin in primary adenocarcinoma cells SW480 (**Figure 4A**, left panels). Similar results were obtained by upregulating LonP1 in I407 and RKO cells (Figures S1A,C in Supplementary Material). In particular, forced LonP1 expression in these cells led to threefold decrease of E-cadherin levels and 2.5-fold increase of β-ctn levels in I407 and RKO cells, respectively. I407-pLonP1 cells also exhibited an elongated, mesenchymal-like morphology, which is typically observed in EMT (Figure S1B in Supplementary Material). Modulation of β-ctn in SW480-pLonP1 cells was also confirmed by fluorescence microscopy (**Figure 4B**). The

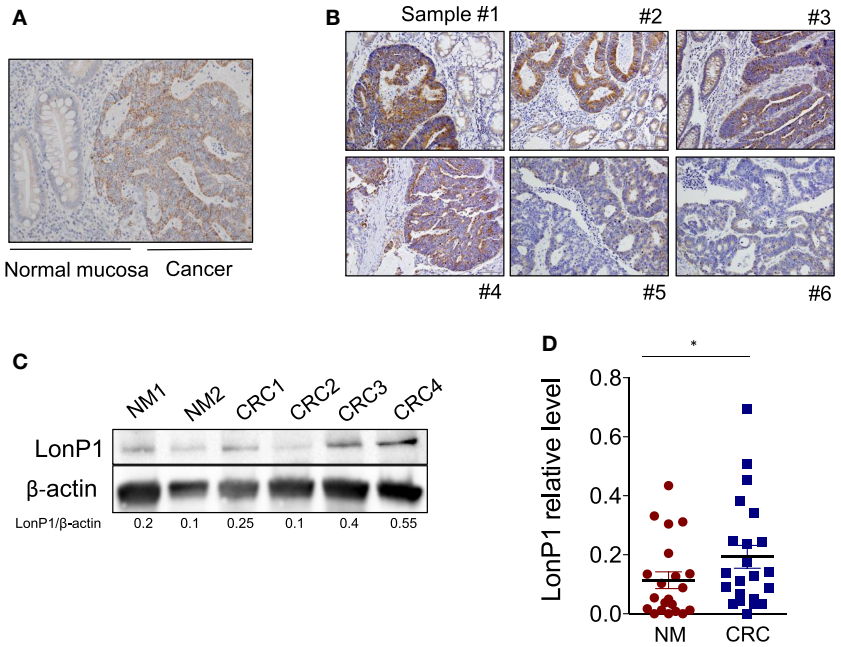


FIGURE 2 | LonP1 is upregulated in CRC tissues. **(A)** Representative immunohistochemical staining of LonP1 in paraffin-embedded human colorectal cancer (CRC) tissue and patient-matched adjacent normal mucosa. **(B)** Representative immunohistochemical stainings of LonP1 high or low level overexpression in paraffin-embedded human CRC tissues. **(C)** Representative Western blot analysis of LonP1 expression in CRC tissues and normal mucosa. **(D)** Relative protein levels of LonP1 are significantly higher in CRC tissues than in adjacent normal mucosa. Data are reported as mean ± SD ($n = 21$); $^*P < 0.05$.

upregulation of LonP1 in SW620 cells, which are metastatic cells, led to opposite results (**Figure 4A**, right panels). Interestingly, downregulation of LonP1 in SW620 cells was associated with reduced β-ctn expression, increased E-cadherin expression, and unchanged N-cadherin in SW620 cells (**Figure 4C**). Considering that Twist is a key regulator of EMT, its expression was analyzed in SW480 cells overexpressing LonP1. Twist levels were almost twofold increased in SW480-pLonP1 cells, if compared to control SW480-pMSCV cells (**Figure 4D**).

Our study confirmed that LonP1 modulation is associated with changes in β-ctn levels and distribution, both *in vitro* and *ex vivo*, in samples from CRC patients. Considering the central role of Akt and GSK-3β in the regulation of β-ctn, we examined the phosphorylation status of Akt and GSK-3β, at serine 473 and serine 9, respectively. We found that LonP1 overexpression led to increased levels of phosphorylated Akt (p-Akt) and phosphorylated GSK-3β (p-GSK-3β), whereas LonP1 depletion led to a slight decrease in p-Akt and p-GSK-3β (**Figures 5A,B**).

LonP1 Impacts Glycolysis in Colon Cancer Cells

As Akt/GSK-3β signaling pathway plays a critical role in regulating glucose metabolism (24), in SW480 and SW620 cells we examined whether forced LonP1 expression had effects on the expression of GLUT-1, LDHA, and G6PD. Plasma membrane GLUT-1 levels increased in SW480 cells overexpressing LonP1 (**Figure 6A**). LDHA levels increased whereas G6PD was almost undetectable in SW480 cells overexpressing LonP1 (**Figure 6B**). In this model, glucose 6-phosphate is likely converted into fructose 6-phosphate, thus entering the glycolytic pathway, rather than being converted into glucose 6-phosphate-gluconolactone, and entering the pentose phosphate pathway. In SW620 cells, both LDHA and G6PD levels increased in the presence of high levels of LonP1. We, therefore, analyzed the glycolytic activity of these cells by performing a real-time analysis of the extracellular acidification rate (ECAR, **Figure 6C**). SW480-pLonP1 cells exhibited a higher maximal glycolysis compared with SW480-pMSCV control cells. Quantitative analysis showed no significant differences in basal glycolysis. No changes were observed in SW620 cells.

Considering that LonP1 has been involved in controlling tumor bioenergetics by reprogramming mitochondrial functions, we analyzed mitochondrial activity by monitoring OCR. Representative curves showed that a difference in mitochondrial respiration is present between SW480-pLonP1 and control cells (**Figure 7A**); quantitative analysis showed that basal OCR was higher in SW480-pLonP1 cells. Basal and coupled respiration as well as maximal respiration and spare respiratory capacity were lower in SW620-pLonP1 cells than in SW620-pMSCV cells (**Figure 7A**). Concerning OCR, slight differences were observed in I407 or RKO cells in the presence of higher levels of LonP1 (Figure S2A in Supplementary Material). Since MMP is required for production of ATP during oxidative phosphorylation (OXPHOS), we analyzed MMP in these cells, by using TMRM. MMP was unchanged in SW480-pLonP1 and I407-pLonP1 cells (**Figure 7A**; Figure S2B in Supplementary Material). However, both SW480-pLonP1 and control cells were sensitive to CCCP-induced MMP depolarization (**Figure 7B**). Interestingly,

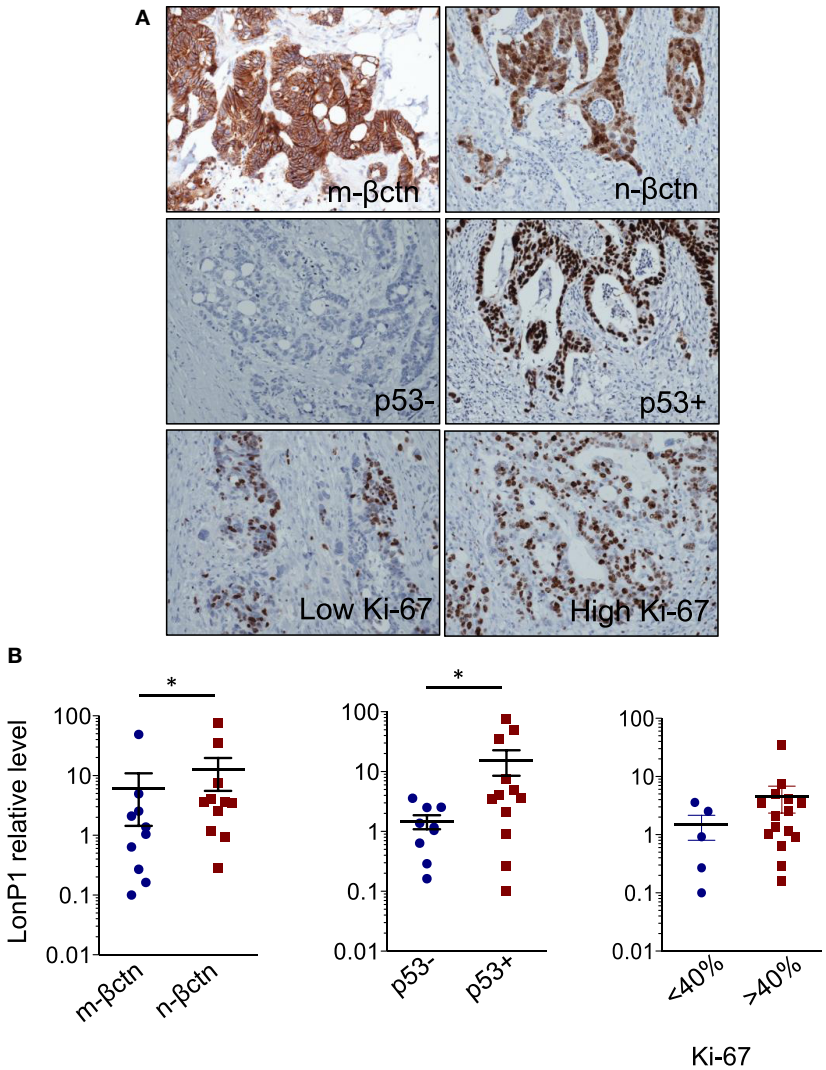


FIGURE 3 | LonP1 upregulation is associated with mutated p53 or nuclear β-ctn. **(A)** Representative immunohistochemical stainings of Ki-67, membrane β-ctn (m-βctn), nuclear β-ctn (n-βctn), and p53 in colorectal cancer (CRC) tissues. **(B)** Association between expression of LonP1, mutated p53, non-mutated p53, membrane β-ctn, or nuclear β-ctn in CRC tissues; *$P < 0.05$.

SW620-pLonP1 and RKO-pLonP1 cells had depolarized mitochondria if compared to SW620-pMSCV and RKO-pMSCV cells, respectively (**Figure 7B**; Figure S2B in Supplementary Material). The reduction of MMP in SW620-pLonP1 was maintained when cells were treated with 10 μM CCCP. As mitochondrial activity is a critical source of ROS, and in particular of anion superoxide, we analyzed its levels by using MitoSOX Red Mitochondrial Superoxide indicator (MitoSOX). We found that SW480-pLonP1 cells produced higher levels of anion superoxide compared to control cells, at the basal level (**Figure 7C**). When challenged with hydrogen peroxide (H_2O_2), no differences were observed between cells overexpressing LonP1 and control cells (**Figure 7C**; Figure S2C in Supplementary Material). Mitochondrial superoxide decreased in SW620-pLonP1 if compared to SW620-pMSCV control cells, both at the basal level and after treatment with H_2O_2 as pro-oxidant stressor (**Figure 7C**).

Finally, we asked whether LonP1 modulation had impact on mitochondrial mass and mitochondrial ultrastructure. Mitochondrial mass was slightly decreased in pLonP1 cells (**Figure 7D**). Analysis of mitochondrial ultrastructure revealed that in SW480-pMSCV and SW480-pLonP1 cells mitochondria were numerous, occupied most of the cytoplasm and displayed *cristae* fragmentation together with the presence of vacuoles and vesicles within the mitochondrial matrix (**Figure 7E**). SW620-pLonP1 cells displayed mitochondrial alterations to a much lesser extent, both in number and in shape, with reduced *cristae* and increased vacuoles, and a noteworthy abundance of free ribosomes in the cytoplasm (**Figure 7E**), reasonably due to a massive synthesis of endogenous proteins, which correlates with particularly aggressive and undifferentiated neoplasms. The counterpart SW620-pMSCV cells did not show altered morphology, or number of mitochondria, whose *cristae* appeared preserved (**Figure 7E**).

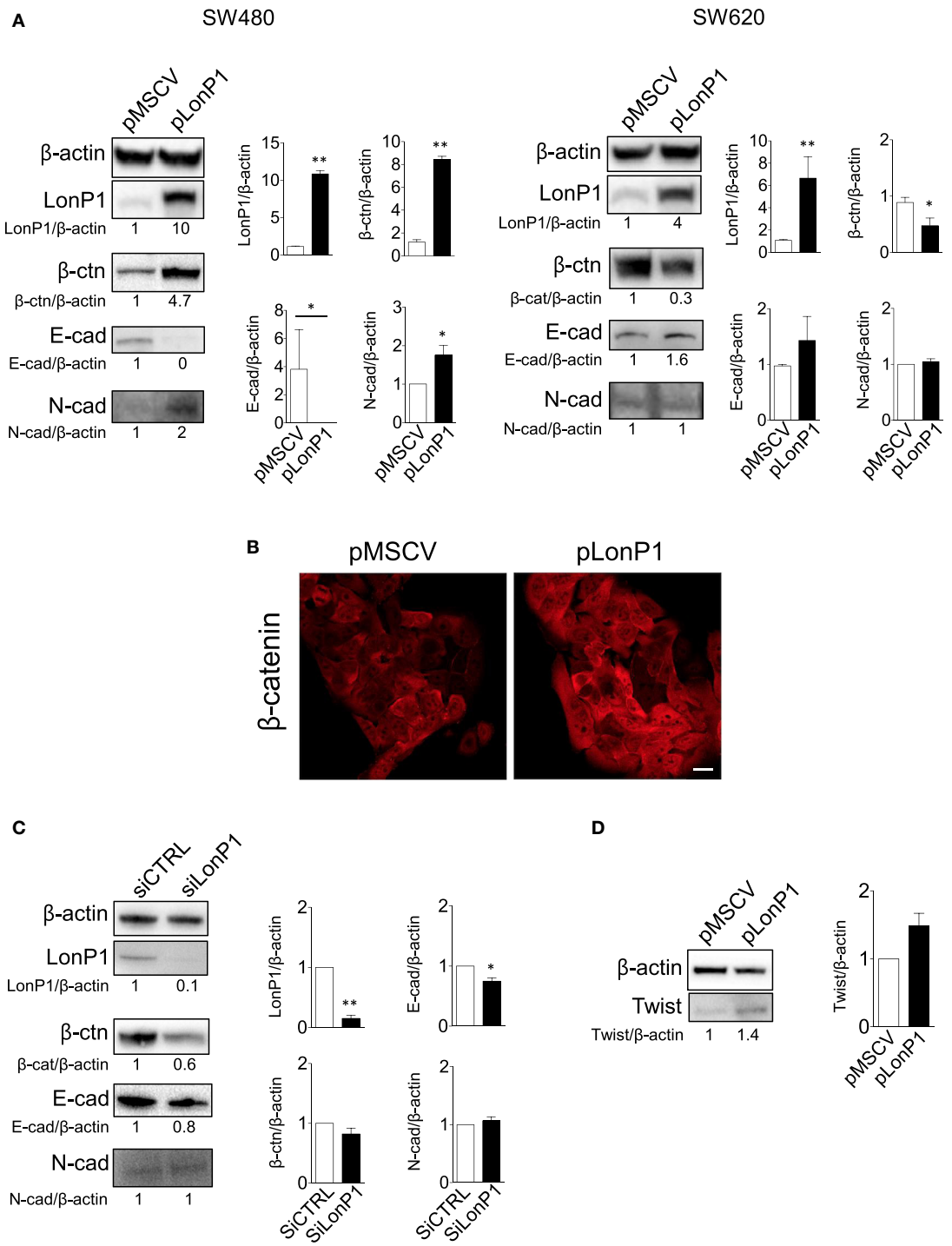


FIGURE 4 | LonP1 modulates E-cadherin, N-cadherin, and β-ctn in SW480 colon primary tumor cells and SW620 metastatic tumor cells. **(A)** Representative Western blot analysis and relative protein level of LonP1, β-catenin (β-ctn), E-cadherin (E-cad), N-cadherin (N-cad) in SW480 and SW620 cells overexpressing LonP1 (namely pLonP1) and in control cells, i.e., cells stably transfected with empty vector (namely pMSCV). β-actin was used as loading control. Densitometries are reported in histograms, and data are reported as mean ± SD ($n = 3$). *$P < 0.05$ and **$P < 0.01$. **(B)** Representative confocal microscopy image showing β-ctn localization in SW480-pMSCV and SW480-pLonP1 cells. Bar, 10 μm. **(C)** Representative Western blot analysis and relative protein level of LonP1, β-ctn, and E-cad SW620, where LonP1 has been downregulated by using small interfering RNAs (siRNAs) against LonP1 mRNA (siLonP1). Control cells were transfected with scramble siRNAs and are indicated as siCTRL. Densitometries are reported in histograms, and data are reported as mean ± SD ($n = 3$). *$P < 0.05$ and **$P < 0.01$. **(D)** Representative Western blot analysis and relative protein level of Twist in SW480 cells overexpressing LonP1 (namely pLonP1) and in control cells, i.e., cells stably transfected with empty vector (namely pMSCV). β-actin was used as loading control. Densitometries are reported in histograms, and data are reported as mean ± SD ($n = 3$).

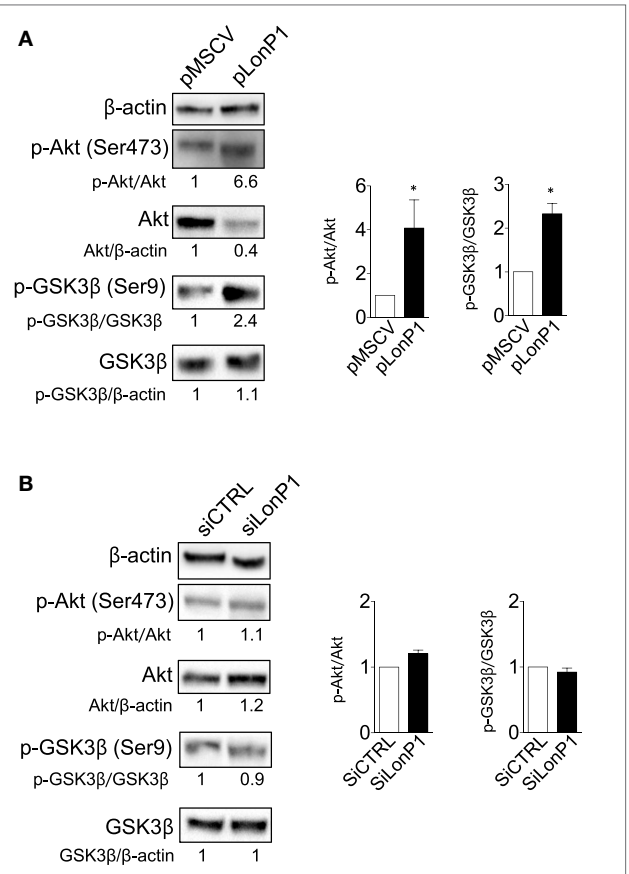


FIGURE 5 | LonP1 modulates β-ctn by regulating Akt/GSK3β signaling. **(A)** Representative Western blot analysis and relative protein levels of phosphorylated Akt (Ser473), Akt, phosphorylated GSK-3β (Ser9), and GSK-3β in SW480 cells overexpressing LonP1. **(B)** Representative Western blot analysis and relative protein levels of phosphorylated Akt (Ser473), Akt, phosphorylated GSK-3β (Ser9), and GSK-3β in SW620 downregulating LonP1. β-actin was used as loading control. Densitometries are reported in histograms, and data are reported as mean ± SD ($n = 3$). *$P < 0.05$.

DISCUSSION

Several studies have detected frequent alterations in the expression of mitochondrial proteases in a variety of human tumors, suggesting that these proteins may play a role as a novel class of tumor promoters or suppressors (14, 25–28). LonP1 is a mitochondrial protease and chaperone located in the mitochondrial matrix. Initial studies placed LonP1 among "stress response proteins," that is those proteins upregulated in response to cell stress (29, 30). More recently, LonP1 has been implicated in the control of metabolic networks in mitochondria in melanoma cells (14), and in hypoxic adaptation in glioma cells (11). We previously showed that: (i) LonP1 is upregulated in several cancer cell lines, including RKO, and in CRC tissues if compared to adjacent normal mucosa, and that (ii) colon cancer cells with low levels of LonP1 displayed reduced levels of OXPHOS complexes, reduced OCR, and increased mitochondrial ROS and highly fragmented and altered mitochondria (22). However, the precise role of LonP1 in colon cancer progression has not been clarified.

In this study, we analyzed the expression of LonP1 in different stages of colon cancer and the consequences of its upregulation at the mitochondrial and cellular level. The first observation is that LonP1 expression gradually increases in normal mucosa, ACF, Ad, and CRC. The fact that LonP1 levels are higher in CRC could suggest a potential role for LonP1 in EMT, an early step in the formation of metastasis (31). We observed high levels of LonP1 in CRC tissues with nuclear localization of β-ctn or in CRC tissues with mutated p53. It is noteworthy that both β-ctn and p53 are directly involved in EMT: β-ctn/TCF4 complex induces EMT through transcription activation of ZEB1 (32), whereas p53 regulates the transcription of genes that are involved in pathways that suppress tumor metastasis, and mutations of p53 can precede metastasis (33). Although previous studies have reported that LonP1 overexpression is associated with decreased E-cadherin, increased N-cadherin and vimentin, a possible link between LonP1 and β-ctn has never been reported (14, 34).

β-ctn is a key component of the Wnt signaling pathway, and acts as negative regulator of E-cadherin in the induction of EMT. In the absence of Wnt stimulation, cytoplasmic β-ctn is phosphorylated by the APC/Axin/GSK-3β complex, and is degraded into the proteasome (35). We reported that LonP1 modulation led to important changes in total β-ctn levels, in several colon cancer cell lines. To understand which pathway was involved in LonP1 regulation of β-ctn, we investigated the phosphorylation status of Akt and GSK-3β, as Akt can phosphorylate, and thus inactivate GSK-3β (36). To explore the role of LonP1 in Akt/GSK-3β signaling pathway, SW480 and SW620 colon carcinoma cell lines were chosen as models since they are derived from primary and secondary tumors resected from the same patient, that makes them a valid tool to investigate changes in colon cancer progression (37). In SW480 cells, the upregulation of an oncoprotein or a protein-like LonP1 would lead to EMT. On the contrary, the downregulation of the same protein in SW620 cells, which are already metastatic, would leave unchanged the mesenchymal phenotype. Indeed, forced LonP1 expression in SW480 led to increased levels of mesenchymal markers, and LonP1 upregulation was associated with increased phosphorylation of Akt and GSK-3β, thus highlighting a role for LonP1 as a regulatory factor in the Wnt/β-ctn pathway. Accordingly, it was interesting to observe that Twist, a key promoter of cancer progression, was involved in EMT. A number of studies have suggested that Twist induces EMT *via* AKT/GSK-3β/β-catenin pathways, among others (38).

While previous studies regarding LonP1 and cancer reported that LonP1 induced EMT through ROS-dependent MAPK signaling, at least in 293 T cells, we found that mitochondrial ROS were not implicated in EMT induction (34). Rather, other mechanisms could be involved, including the alteration of pathways involved in the rearrangement of cellular metabolism. It has been reported that LonP1 controls tumor bioenergetics by remodeling subunits of electron transport chain (14). We found that in colon cancer cells LonP1 can influence glycolytic

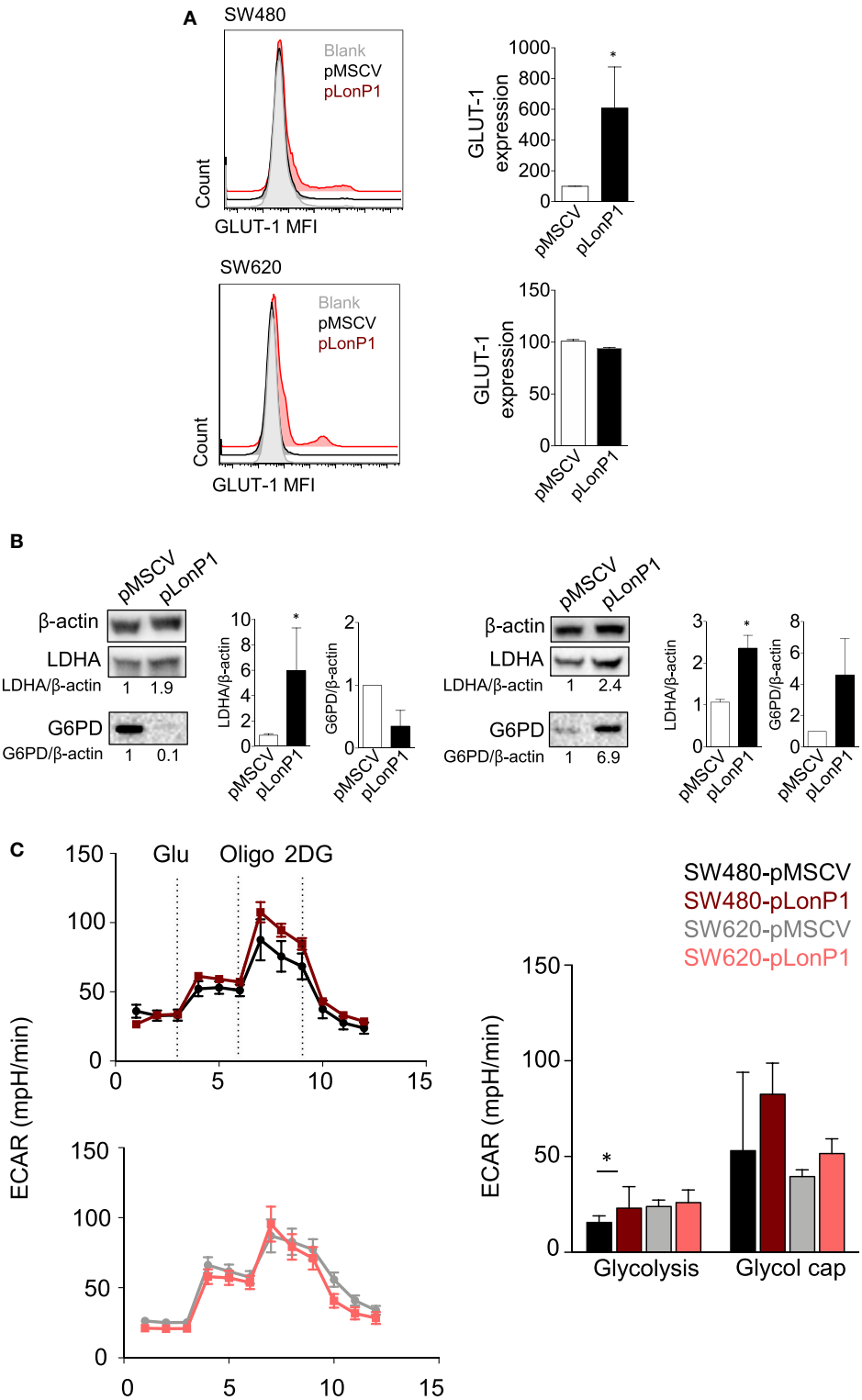


FIGURE 6 | LonP1 modulates glycolytic activity in colon cancer cells. **(A)** Representative histograms showing glucose transporter (GLUT)-1 expression in SW480-pMSCV, SW480-pLonP1, SW620-pMSCV, and SW620-pLonP1 cells. Quantitative analysis is reported on the right and shows the median fluorescence intensity of plasma membrane GLUT-1 levels, as revealed by flow cytometry. **(B)** Representative Western blot analysis and relative protein levels of phosphorylated lactate dehydrogenase A and glucose 6-phosphate dehydrogenase in SW480 and SW620 cells overexpressing LonP1. β-actin was used as loading control. Densitometries are reported in histograms, and data are reported as mean ± SD ($n = 3$). *$P < 0.05$. **(C)** Representative traces and quantitative analysis of extracellular acidification rate in indicated cells. Subsequent injections of glucose (Glu), oligomycin (Oligo), and 2-deoxy-glucose were performed as indicated.

activity together with mitochondrial activity. Alongside, effects of LonP1 overexpression have been observed in the initial steps of pentose phosphate pathway, which is required for the synthesis of ribonucleotides from glucose and is a major source of nicotinamide adenine dinucleotide phosphate. Pentose pathway is important for redox balance and anabolism, and seems indeed to be promoted in SW620-pLonP1 cells. In SW620-pLonP1 cells, glucose could be redistributed to alternative pathways, such as the pentose pathway, to support growth and survival of these cells, which were characterized by reduced OXPHOS. In agreement with this observation, the pentose phosphate pathway was strongly decreased in SW480-pLonP1, where OXPHOS was almost unchanged. Despite the fact that SW480 and SW620 have the same genetic background, characterized by mutations in KRAS and TP53, overexpression of LonP1 has dramatically different consequences on mitochondrial function, bioenergetics, and in malignant transformation. While LonP1 does not influence OCR or mitochondrial functions in SW480 cells, its overexpression in SW620 cells determines a reduction of OCR and depolarization of mitochondrial membrane. We would expect that mitochondrial membrane depolarization resulted in increased ROS production, but this was not case. We rather observed a decrease in mitochondrial anion superoxide in SW620-pLonP1 when compared with SW620-pMSCV cells. The analysis of morphology and ultrastructure of mitochondria confirmed that overexpression of LonP1 has a stronger impact on SW620 than SW480 cells.

In conclusion, our findings demonstrate a role for LonP1 in the regulation of EMT *via* GSK-3β/β-ctn and modifications in cellular metabolism, suggesting that changes in LonP1 expression and EMT in CRC are likely not two independent, concurrent phenomena, but might be functionally linked.

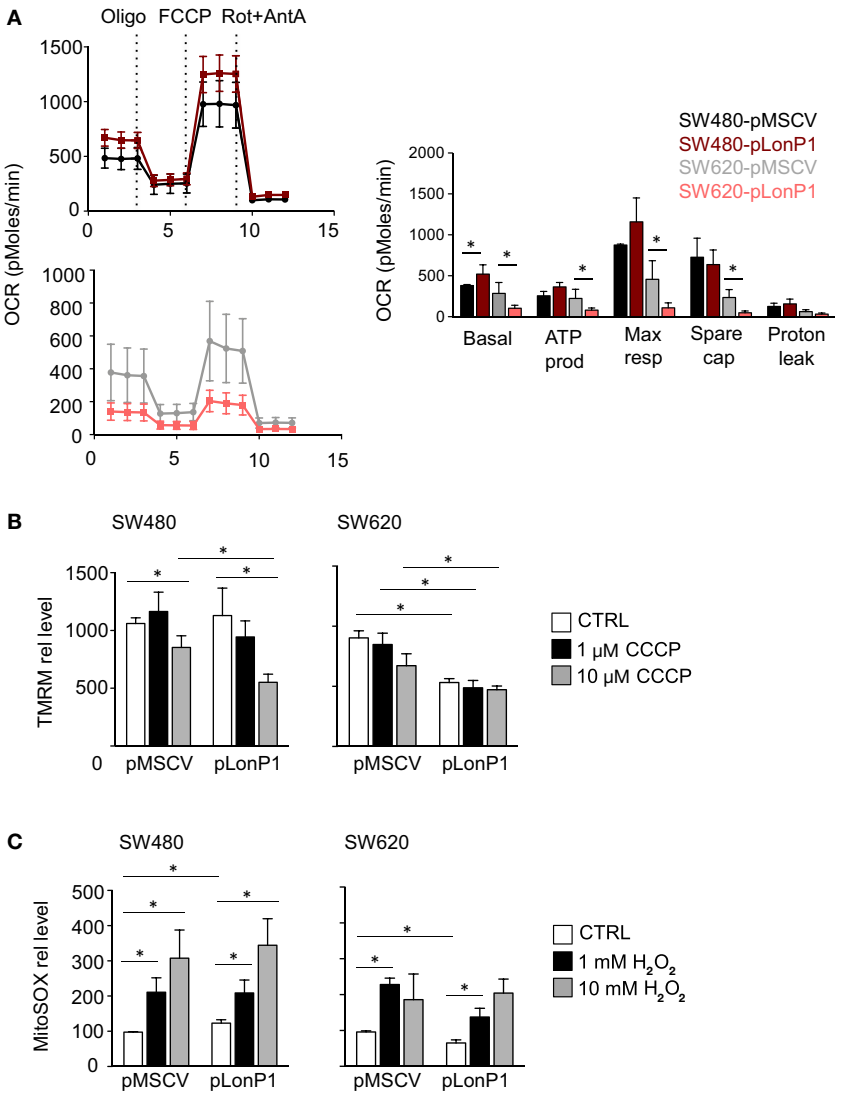


FIGURE 7 | Continued

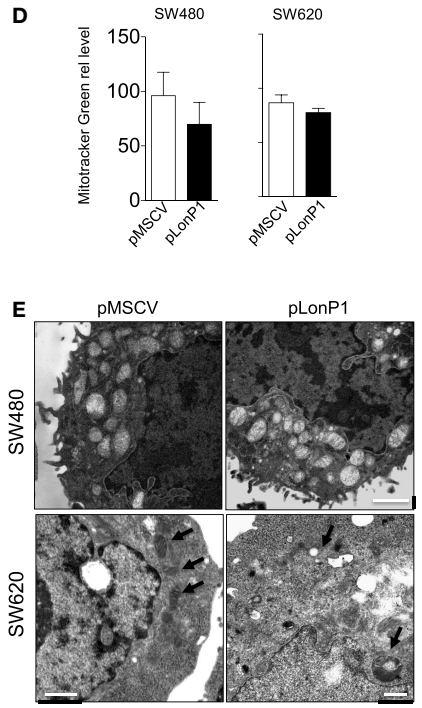


FIGURE 7 | LonP1 slightly modulates mitochondrial activity in colon cancer cells. **(A)** Representative traces and quantitative analysis of the oxygen consumption rate in indicated cells. Subsequent injections of oligomycin (Oligo), mitochondrial decoupler carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), complex I inhibitor rotenone (Rot), and complex III inhibitor (AntA) were performed as indicated; $^{*}P < 0.05$. **(B)** Mitochondrial membrane potential quantification was assayed by tetramethyl rodhamine methyl esther (TMRM) in the presence or absence of CCCP in indicated cells. Data are expressed as percentage of increase in median fluorescence intensity (MFI) and represented the mean ± SD ($n = 4$); $^{*}P < 0.05$. **(C)** Mitochondrial anion superoxide quantification as assayed by MitoSOX Red Mitochondrial Superoxide Indicator (mitoSOX) in the presence or absence of hydrogen peroxide in indicated cells. Data are expressed as percentage of increase in MFI and represented the mean ± SD ($n = 4$). **(D)** Mitochondrial mass quantification as assayed by Mitotracker Green in indicated cells. Data are expressed as percentage of increase in MFI and represented the mean ± SD ($n = 4$); $^{*}P < 0.05$. **(E)** Representative transmission electron microscopy images of indicated cells. Scale bars, 1 μm.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Ethical Committee of the province of Modena (Italy). The protocol was approved by the Ethical Committee of the province of Modena. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

LG, AC, and MP conceived and designed the experiments. LG, SB, DT, GC, AP, AG, SPe, and SPa conducted the experiments. LL, FM, and LR provided samples and performed immunohistochemical stainings. MN reported and organized data. LG, LL, AI, PP, AC, and MP wrote and revised the paper.

ACKNOWLEDGMENTS

This work was supported by grant from "Associazione Angela Serra per la Ricerca sul Cancro" (Modena) to AC and by "Associazione Italiana Ricerca sul Cancro" (AIRC, Investigator Grant number 19786) to MP. We gratefully acknowledged the "Associazione Italiana Mogli Medici" in Modena for the kind donation of the Trans-Blot Turbo Blotting System to AC. Sara De Biasi is an International Society for Advancement in Cytometry (ISAC) Marylou Ingram Scholar.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fonc.2018.00254/full#supplementary-material.

FIGURE S1 | LonP1 modulates E-cadherin, N-cadherin, and β-ctn in I407 and RKO cells. **(A)** Representative Western blot analysis and relative protein level of LonP1, β-catenin (β-ctn), E-cadherin (E-cad), N-cadherin (N-cad) in I407 cells overexpressing LonP1 (namely pLonP1) and in control cells, i.e., cells stably transfected with empty vector (namely pMSCV). β-actin was used as loading control. Densitometries are reported in histograms, and data are reported as mean ± SD ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$. **(B)** Representative phase contrast images of I407 cells overexpressing LonP1 (pLonP1) and control cells (pMSCV). Scale bar, 10 μm. **(C)** Representative Western blot analysis and relative protein level of LonP1, β-catenin (β-ctn), E-cadherin (E-cad), and N-cadherin (N-cad) in RKO cells overexpressing LonP1 (namely pLonP1) and in control cells, i.e., cells stably transfected with empty vector (namely pMSCV). β-actin was used as loading control. Densitometries are reported in histograms, and data are reported as mean ± SD ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$.

FIGURE S2 | LonP1 slightly modulates mitochondrial activity in I407 and RKO colon cancer cells. **(A)** Representative traces and quantitative analysis of the oxygen consumption rate in indicated cells. Subsequent injections of oligomycin (Oligo), mitochondrial decoupler carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), complex I inhibitor rotenone (Rot) and complex III inhibitor (AntA) were performed as indicated. **(B)** Mitochondrial membrane potential quantification as assayed by tetramethyl rodhamine methyl esther (TMRM) in the presence or absence of CCCP in indicated cells. Data are expressed as percentage of increase in median fluorescence intensity (MFI) and represented the mean ± SD ($n = 3$); $^*P < 0.05$. **(C)** Mitochondrial anion superoxide quantification as assayed by MitoSOX Red Mitochondrial Superoxide Indicator (mitoSOX) in the presence or absence of hydrogen peroxide in indicated cells. Data are expressed as percentage of increase in MFI and represented the mean ± SD (n = 3); $^*P < 0.05$.

REFERENCES

1. Pinti M, Gibellini L, Liu Y, Xu S, Lu B, Cossarizza A. Mitochondrial Lon protease at the crossroads of oxidative stress, ageing and cancer. *Cell Mol Life Sci* (2015) 72:4807–24. doi:10.1007/s00018-015-2039-3
2. Bota DA, Davies KJ. Lon protease preferentially degrades oxidized mitochondrial aconitase by an ATP-stimulated mechanism. *Nat Cell Biol* (2002) 4:674–80. doi:10.1038/ncb836
3. Fukuda R, Zhang H, Kim JW, Shimoda L, Dang CV, Semenza GL. HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells. *Cell* (2007) 129:111–22. doi:10.1016/j.cell.2007.01.047
4. Granot Z, Kobiler O, Melamed-Book N, Eimerl S, Bahat A, Lu B, et al. Turnover of mitochondrial steroidogenic acute regulatory (StAR) protein by Lon protease: the unexpected effect of proteasome inhibitors. *Mol Endocrinol* (2007) 21:2164–77. doi:10.1210/me.2005-0458
5. Kita K, Suzuki T, Ochi T. Diphenylarsinic acid promotes degradation of glutaminase C by mitochondrial Lon protease. *J Biol Chem* (2012) 287:18163–72. doi:10.1074/jbc.M112.362699
6. Lu B, Lee J, Nie X, Li M, Morozov YI, Venkatesh S, et al. Phosphorylation of human TFAM in mitochondria impairs DNA binding and promotes degradation by the AAA+ Lon protease. *Mol Cell* (2013) 49:121–32. doi:10.1016/j.molcel.2012.10.023
7. Teng H, Wu B, Zhao K, Yang G, Wu L, Wang R. Oxygen-sensitive mitochondrial accumulation of cystathionine beta-synthase mediated by Lon protease. *Proc Natl Acad Sci U S A* (2013) 110:12679–84. doi:10.1073/pnas.1308487110
8. Tian Q, Li T, Hou W, Zheng J, Schrum LW, Bonkovsky HL. Lon peptidase 1 (LONP1)-dependent breakdown of mitochondrial 5-aminolevulinic acid synthase protein by heme in human liver cells. *J Biol Chem* (2011) 286:26424–30. doi:10.1074/jbc.M110.215772
9. Friguet B, Bulteau AL, Petropoulos I. Mitochondrial protein quality control: implications in ageing. *Biotechnol J* (2008) 3:757–64. doi:10.1002/biot.200800041
10. Ngo JK, Davies KJ. Importance of the lon protease in mitochondrial maintenance and the significance of declining lon in aging. *Ann N Y Acad Sci* (2007) 1119:78–87. doi:10.1196/annals.1404.015
11. Di K, Lomeli N, Wood SD, Vanderwal CD, Bota DA. Mitochondrial Lon is over-expressed in high-grade gliomas, and mediates hypoxic adaptation: potential role of Lon as a therapeutic target in glioma. *Oncotarget* (2016) 7:77457–67. doi:10.18632/oncotarget.12681
12. Liu Y, Lan L, Huang K, Wang R, Xu C, Shi Y, et al. Inhibition of Lon blocks cell proliferation, enhances chemosensitivity by promoting apoptosis and decreases cellular bioenergetics of bladder cancer: potential roles of Lon as a prognostic marker and therapeutic target in baldder cancer. *Oncotarget* (2014) 5:11209–24. doi:10.18632/oncotarget.2026
13. Luo B, Wang M, Hou N, Hu X, Jia G, Qin X, et al. ATP-dependent Lon protease contributes to *Helicobacter pylori*-induced gastric carcinogenesis. *Neoplasia* (2016) 18:242–52. doi:10.1016/j.neo.2016.03.001
14. Quiros PM, Espanol Y, Acin-Perez R, Rodriguez F, Barcena C, Watanabe K, et al. ATP-dependent Lon protease controls tumor bioenergetics by reprogramming mitochondrial activity. *Cell Rep* (2014) 8:542–56. doi:10.1016/j.celrep.2014.06.018
15. Gibellini L, Pinti M, Bartolomeo R, De Biasi S, Cormio A, Musicco C, et al. Inhibition of Lon protease by triterpenoids alters mitochondria and is associated to cell death in human cancer cells. *Oncotarget* (2015) 6:25466–83. doi:10.18632/oncotarget.4510
16. Gibellini L, Pinti M, Beretti F, Pierri CL, Onofrio A, Riccio M, et al. Sirtuin 3 interacts with Lon protease and regulates its acetylation status. *Mitochondrion* (2014) 18:76–81. doi:10.1016/j.mito.2014.08.001
17. Siegel R, Desantis C, Jemal A. Colorectal cancer statistics, 2014. *CA Cancer J Clin* (2014) 64:104–17. doi:10.3322/caac.21220
18. Brenner H, Kloor M, Pox CP. Colorectal cancer. *Lancet* (2014) 383:1490–502. doi:10.1016/S0140-6736(13)61649-9
19. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest* (2009) 119:1420–8. doi:10.1172/JCI39104
20. White BD, Chien AJ, Dawson DW. Dysregulation of Wnt/beta-catenin signaling in gastrointestinal cancers. *Gastroenterology* (2012) 142:219–32. doi:10.1053/j.gastro.2011.12.001
21. Moon RT, Kohn AD, De Ferrari GV, Kaykas A. WNT and beta-catenin signalling: diseases and therapies. *Nat Rev Genet* (2004) 5:691–701. doi:10.1038/nrg1427
22. Gibellini L, Pinti M, Boraldi F, Giorgio V, Bernardi P, Bartolomeo R, et al. Silencing of mitochondrial Lon protease deeply impairs mitochondrial proteome and function in colon cancer cells. *FASEB J* (2014) 28:5122–35. doi:10.1096/fj.14-255869
23. Gibellini L, De Biasi S, Pinti M, Nasi M, Riccio M, Carnevale G, et al. The protease inhibitor atazanavir triggers autophagy and mitophagy in human preadipocytes. *AIDS* (2012) 26:2017–26. doi:10.1097/QAD.0b013e328359b8be
24. Zhao Y, Hu X, Liu Y, Dong S, Wen Z, He W, et al. ROS signaling under metabolic stress: cross-talk between AMPK and AKT pathway. *Mol Cancer* (2017) 16:79. doi:10.1186/s12943-017-0648-1
25. Cole A, Wang Z, Coyaud E, Voisin V, Gronda M, Jitkova Y, et al. Inhibition of the mitochondrial protease ClpP as a therapeutic strategy for human acute myeloid leukemia. *Cancer Cell* (2015) 27:864–76. doi:10.1016/j.ccell.2015.05.004
26. Pinti M, Gibellini L, Nasi M, De Biasi S, Bortolotti CA, Iannone A, et al. Emerging role of Lon protease as a master regulator of mitochondrial functions. *Biochim Biophys Acta* (2016) 1857(8):1300–6. doi:10.1016/j.bbabio.2016.03.025
27. Seo JH, Rivadeneira DB, Caino MC, Chae YC, Speicher DW, Tang HY, et al. The mitochondrial unfoldase-peptidase complex ClpXP controls bioenergetics stress and metastasis. *PLoS Biol* (2016) 14:e1002507. doi:10.1371/journal.pbio.1002507
28. Yamauchi S, Hou YY, Guo AK, Hirata H, Nakajima W, Yip AK, et al. p53-mediated activation of the mitochondrial protease HtrA2/Omi prevents cell invasion. *J Cell Biol* (2014) 204:1191–207. doi:10.1083/jcb.201309107
29. Pinti M, Gibellini L, De Biasi S, Nasi M, Roat E, O'Connor JE, et al. Functional characterization of the promoter of the human Lon protease gene. *Mitochondrion* (2011) 11:200–6. doi:10.1016/j.mito.2010.09.010
30. Pinti M, Gibellini L, Guaraldi G, Orlando G, Gant TW, Morselli E, et al. Upregulation of nuclear-encoded mitochondrial LON protease in HAART-treated HIV-positive patients with lipodystrophy: implications for the pathogenesis of the disease. *AIDS* (2010) 24:841–50. doi:10.1097/QAD.0b013e32833779a3
31. Puisieux A, Brabletz T, Caramel J. Oncogenic roles of EMT-inducing transcription factors. *Nat Cell Biol* (2014) 16:488–94. doi:10.1038/ncb2976
32. Sanchez-Tillo E, de Barrios O, Siles L, Cuatrecasas M, Castells A, Postigo A. beta-catenin/TCF4 complex induces the epithelial-to-mesenchymal transition (EMT)-activator ZEB1 to regulate tumor invasiveness. *Proc Natl Acad Sci U S A* (2011) 108:19204–9. doi:10.1073/pnas.1108977108
33. Powell E, Piwnica-Worms D, Piwnica-Worms H. Contribution of p53 to metastasis. *Cancer Discov* (2014) 4:405–14. doi:10.1158/2159-8290.CD-13-0136
34. Cheng CW, Kuo CY, Fan CC, Fang WC, Jiang SS, Lo YK, et al. Overexpression of Lon contributes to survival and aggressive phenotype of cancer cells through mitochondrial complex I-mediated generation of reactive oxygen species. *Cell Death Dis* (2013) 4:e681. doi:10.1038/cddis.2013.204
35. Clevers H. Wnt/beta-catenin signaling in development and disease. *Cell* (2006) 127:469–80. doi:10.1016/j.cell.2006.10.018
36. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* (1995) 378:785–9. doi:10.1038/378785a0
37. Hewitt RE, McMarlin A, Kleiner D, Wersto R, Martin P, Tsokos M, et al. Validation of a model of colon cancer progression. *J Pathol* (2000) 192:446–54. doi:10.1002/1096-9896(2000)9999:9999<::AID-PATH775>3.0.CO;2-K

38. Oh BY, Kim SY, Lee YS, Hong HK, Kim TW, Kim SH, et al. Twist1-induced epithelial-mesenchymal transition according to microsatellite instability status in colon cancer cells. *Oncotarget* (2016) 7:57066–76. doi:10.18632/oncotarget.10974

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered as potential conflict of interest.

frontiers in Oncology

REVIEW
published: 23 April 2018
doi: 10.3389/fonc.2018.00126

Signaling Pathways Regulating Redox Balance in Cancer Metabolism

Maria Chiara De Santis[1], Paolo Ettore Porporato[1], Miriam Martini[1] and Andrea Morandi[2]*

[1] Department of Molecular Biotechnology and Health Science, Molecular Biotechnology Center, University of Torino, Torino, Italy, [2] Department of Experimental and Clinical Biomedical Sciences, University of Florence, Florence, Italy

The interplay between rewiring tumor metabolism and oncogenic driver mutations is only beginning to be appreciated. Metabolic deregulation has been described for decades as a bystander effect of genomic aberrations. However, for the biology of malignant cells, metabolic reprogramming is essential to tackle a harsh environment, including nutrient deprivation, reactive oxygen species production, and oxygen withdrawal. Besides the well-investigated glycolytic metabolism, it is emerging that several other metabolic fluxes are relevant for tumorigenesis in supporting redox balance, most notably pentose phosphate pathway, folate, and mitochondrial metabolism. The relationship between metabolic rewiring and mutant genes is still unclear and, therefore, we will discuss how metabolic needs and oncogene mutations influence each other to satisfy cancer cells' demands. Mutations in oncogenes, i.e., PI3K/AKT/mTOR, RAS pathway, and MYC, and tumor suppressors, i.e., p53 and liver kinase B1, result in metabolic flexibility and may influence response to therapy. Since metabolic rewiring is shaped by oncogenic driver mutations, understanding how specific alterations in signaling pathways affect different metabolic fluxes will be instrumental for the development of novel targeted therapies. In the era of personalized medicine, the combination of driver mutations, metabolite levels, and tissue of origins will pave the way to innovative therapeutic interventions.

Keywords: metabolic reprogramming, one-carbon metabolism, OXPHOS, oncometabolites, pentose phosphate pathway

OPEN ACCESS

Edited by:
Andrea Rasola, Università degli Studi di Padova, Italy

Reviewed by:
Marco Sciacovelli, University of Cambridge, United Kingdom
Stefano Indraccolo, Istituto Oncologico Veneto (IRCCS), Italy

****Correspondence:***
Miriam Martini
miriam.martini@unito.it

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *31 January 2018*
Accepted: *06 April 2018*
Published: *23 April 2018*

Citation:
De Santis MC, Porporato PE, Martini M and Morandi A (2018) Signaling Pathways Regulating Redox Balance in Cancer Metabolism. Front. Oncol. 8:126. doi: 10.3389/fonc.2018.00126

INTRODUCTION: METABOLIC DEREGULATION AND TUMOR PROGRESSION

Despite being considered for decades as a bystander effect of the genomic alterations that characterize a cancer cell, it is now established that the deregulation of key metabolic hubs can be a tumorigenic driver and that several cellular signaling alterations converge toward metabolic

Abbreviations: 1-CM, one-carbon metabolism; ALDH, aldehyde dehydrogenase 1 family member; αKG, alpha-ketoglutarate; DRP1, dynamin-related protein; F6P, fructose-6-phosphate; FH, fumarate hydratase; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; IDH, isocitrate dehydrogenase; MDM2, mouse double minute 2 homolog; MOMP, mitochondrial outer membrane permeabilization; MTHFD, methylenetetrahydrofolate dehydrogenase (NADP + dependent); NADPH, nicotinamide adenine dinucleotide phosphate; OMM, outer mitochondria membrane; PGAM1, phosphoglycerate mutase 1; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1 alpha; PHGDH, phosphoglycerate dehydrogenase; SDH, succinate dehydrogenase; SSP, serine synthesis pathway; TCA, tricarboxylic acid; TIGAR, TP53-induced glycolysis and apoptosis regulator; PPP, pentose phosphate pathway; ROS, reactive oxygen species.

alterations and the accumulation of specific intermediates endowed with oncogenic potential (1). The altered metabolic scenario of a cancer cell inevitably affects its cellular redox homeostasis. Particularly, cancer cells are characterized by an altered redox status and enhanced reactive oxygen species (ROS) that have been shown to drive and sustain cancer cells proliferation. Higher ROS levels are compensated by an increase in antioxidant mechanism that allows the cancer cell to survive in a pro-oxidant environment. However, such pro-oxidant condition favors DNA damage and genomic instability, events that concur to enhance the malignant traits of cancer cells, including metabolic reprogramming. Metabolic reprogramming and redox homeostasis are intimately interconnected and, therefore, such a vicious loop is further promoted by genetic lesions that activate proto-oncogenes or repress onco-suppressors, leading to cancer progression (2).

This review gathers the recent findings on the role of oncogenic-dependent metabolic reprogramming that cancer cells undergo during the different stages of tumor progression. Importantly, the main metabolic pathways concurring to redox homeostasis have been reviewed with a focus on the correlation between oncogenic lesions and ROS-dependent metabolic reprogramming.

PENTOSE PHOSPHATE PATHWAY (PPP)

The PPP, also termed phosphogluconate pathway, diverges from the glycolytic pathway after the initial phosphorylation of glucose catalyzed by the glycolytic enzyme hexokinase. Glucose-6-phosphate (G6P) is the principal substrate of the G6P dehydrogenase (G6PDH), the rate-limiting enzyme of the PPP. The primary endpoint of the PPP is to provide (i) phospho-pentoses, for the nucleotides and nucleic acids synthesis and (ii) reducing equivalents that are used for both reductive biosynthesis reactions and for redox homeostasis. Indeed, the production of reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the oxidative phase of the pathway is essential for redox cellular homeostasis; directly, by buffering enhanced reactive oxygen intermediates and indirectly for the regeneration of the oxidized form of the glutathione (GSH), a pivotal molecule in neutralizing intracellular ROS levels (3). The non-oxidative phase of the PPP is characterized by a series of reversible reactions catalyzed by transaldolase (TALDO) and transketolase (TKT) enzymes that recruit additional glycolytic intermediates, i.e., fructose-6-phosphate (F6P) and glyceraldehyde-3-phosphate (G3P), to produce phospho-pentoses (4).

Despite leading to glucose oxidation, the main role of the PPP is anabolic rather than catabolic. Depending on the cellular requirements and response to exogenous stimuli, the PPP and the glycolysis cooperate to provide the needed metabolites. For instance, rapidly proliferating cells will boost the PPP to meet the requirements for pentoses to support nucleotide biosynthesis, both from G6P (oxidative phase) and from F6P and G3P (non-oxidative phase). While this condition is representative of a rapidly dividing cancer cell, a cell that has to maintain the redox cellular balance will sustain the oxidative branch for NADPH production and re-direct the non-oxidative branch toward F6P synthesis from the phospho-pentoses, which are then regenerating G6P to replenish the oxidative branch. ROS accumulation and subsequent oxidative stress result from the unbalance between ROS generation (e.g., from electron transport chain), and antioxidant mechanism (e.g., GSH, thioredoxin, and catalase) requiring NADPH to function as ROS scavengers. Therefore, not surprisingly, PPP deregulation has been linked to the pathogenesis of several diseases, such as G6PDH-deficiency. G6PDH-deficiency, and subsequent reduced activity, impairs the ability of erythrocytes to generate NADPH, hence exposing the cells' phospholipid bilayer to the detrimental effect of ROS, leading to hemolytic anemia. G6PDH-deficiency is not associated with acquired susceptibility to particular diseases and preventing oxidative stress-inducing situations (i.e., certain drugs and food) leaves G6PDH-deficiency bearing individuals asymptomatic. Interestingly, G6PDH-deficiency is protective against malaria, heart, and cerebrovascular disease (5).

However, since the focus of this review is on cancer, we will discuss how PPP deregulation is affected by oncogenic-dependent metabolic reprogramming and how this impacts on tumor progression and therapy resistance. Interestingly, G6PDH-deficiency has been reported to reduce cancer susceptibility and incidence. A retrospective observational study in ~4,000 patients that underwent colonoscopy with a 10 years follow-up from Sardinia region (Italy), where G6PDH-deficiency prevalence ranges between 12 and 24% and is often caused by the $G6PDH^{C563T}$ variant, shows that G6PDH-deficiency is associated with reduced colorectal cancer risk (5, 6). The potential explanation of such counterintuitive epidemiological data may be explained by the NADPH and PPP dependence that sustain lipid anabolism in cancer cells. Additionally, NADPH deficiency due to G6PDH alterations may alter the redox balance, hence reducing intracellular ROS levels, which play an important role in cancer initiation by increasing the DNA mutation rate and the synthesis of proinflammatory cytokines (7).

Glucose-6-phosphate dehydrogenase expression and activity have been proposed to be importantly influenced by genetic alterations that occur on oncogenes and/or oncosuppressor that ultimately lead to a pro-mitogenic signaling pathways activation of the cancer cells (**Figure 1**). Indeed, while the reprogramming of certain metabolic hubs seems to correlate with particular tumor phenotype and be associated with certain type of therapeutic option, the increased demand of NADPH, used for either antioxidant response and/or cellular anabolism than pentoses, is a prerequisite of almost any cancer (4). G6PDH is regulated by a plethora of extracellular stimuli, e.g., growth factors, that impacts on its expression and activity *via* the MAPK and PI3K signaling pathways. Since these signaling pathways are often hyper-activated in cancer due to oncogenes activation (e.g., K-RAS, MYC, and growth factor receptors), or oncosuppressors inactivation (e.g., p53 and PTEN loss-of-function and inactivation), any alterations that impact on these players may lead to G6PDH enhanced expression and activity (8, 9). Additionally, some of the aforementioned cancer-inducing genes can also regulate G6PDH function independently of the signaling cascade. For instance, loss of p53, which directly controls and inhibits glucose transporters expression (10), leads to enhanced glucose uptake that can be diverted into the PPP pathway. Similarly, glucose diversion

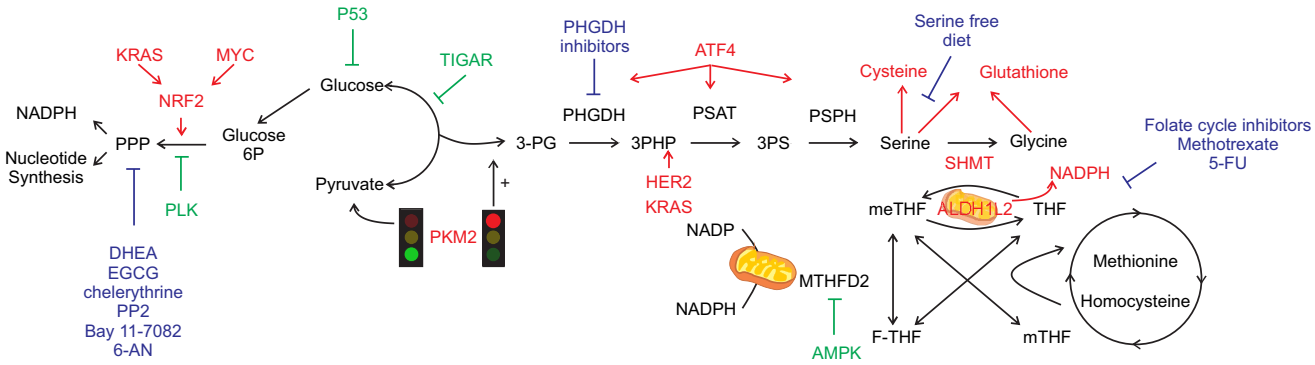


FIGURE 1 | One-carbon metabolism (1-CM) and pentose phosphate pathway (PPP) at the cross-road between anabolism and redox balance. Simplified scheme of the crosstalk between metabolic and signaling pathways that can occur in cancer cells. Pro-tumorigenic signals are represented in red and anti-tumorigenic in green. Glycolysis regulates both PPP and 1-CM. Serine signaling pathway, once PKM2 activity is inhibited (red light), leads to glycolytic intermediates accumulation hence fueling both PPP and 1-CM. Serine metabolism enzymes bridge the shunt between glycolysis and 1-CM, with specific oncogene upregulating the enzymes involved [i.e., phosphoglycerate dehydrogenase (PGHDH), phosphoserine aminotransferase 1 (PSAT1), phosphoserine phosphatase (PSPH)], leading to serine generation and antioxidant machinery intermediates (cysteine and glutathione). Serine hydroxymethyltransferase (SHMT) mediates the conversion between serine and glycine with the concurrent transformation of tetrahydrofolate (THF) into 5,10-methylenetetrahydrofolate (meTHF). Aldehyde dehydrogenase 1 family member (ALDH1L) mediates the opposite reaction with concurrent generation of NADPH, similarly methylenetetrahydrofolate dehydrogenase (MTHDF) transform meTHF into 10-formyltetrahydrofolate (F-THF) with concurrent generation of nicotinamide adenine dinucleotide phosphate. The main chemotherapy agents are represented in blue. Dehydroepiandrosterone (DHEA), epigallocatechin gallate (EGCG), amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2), 6-aminonicotinamide (6-AN), and 5-fluorouracil (5-FU).

into the PPP can also be a consequence of the downregulation of TP53-induced glycolysis and apoptosis regulator (TIGAR), a p53-dependent gene known to inhibit the glycolytic enzyme phosphoglycerate mutase 1 (PGAM1) that leads to the increase of the oxidative PPP by reducing the amount of 3-phosphoglycerate (3PG), which has been reported to inhibit the PPP enzyme 6-phosphogluconate dehydrogenase (6PGDH) (11). Finally, p53-mediated G6PDH inhibition can also be mediated by direct protein-to-protein contact (3). DNA damage, telomeric instability, or oxidative stress can activate the ataxia telangiectasia-mutated (ATM) kinase, which in turn activates p53. Interestingly, ATM promotes the Hsp27 phosphorylation and binding to G6PDH, stimulating its activity and, therefore, increasing the PPP flux and the subsequent NADPH and phospho-pentoses production (12). However, others have reported that loss of p53 enhances NADPH production, therefore, by generating a negative feedback on the PPP. These reported contradictory results may be related to the different role that p53 can exert on either cell cycle or apoptosis (3). Finally, it has been recently demonstrated that polo-like kinase 1 (PLK1), a key player in cell mitosis, is able to directly phosphorylate and activate G6PDH (13).

Although G6PDH is the major rate-limiting step of the PPP, the amount of phosphorylated glucose trapped into the cells and the expression of enzymes regulating the glycolytic rate can also affect the PPP flux. Indeed, higher levels of G6P are required to sustain oxidative and non-oxidative branches of the PPP and this can be achieved by (i) enhancing the amount of glucose that is phosphorylated by the glycolytic-rate limiting enzyme hexokinase 2 (HK2), the main isoform expressed by cancer cells, and (ii) by engulfing the glycolytic pathway and subsequent enhancing the accumulation of glycolytic intermediates. HK2 enhanced expression, known to be regulated by oncogenic RAS activation, is essential to sustain the non-oxidative phase of the PPP in lung cancer (14). Additionally, pyruvate kinase (PK)-M2, an isoform of the PK that converts phosphoenolpyruvate (PEP) into pyruvate and that is expressed by many cancers, can be controlled by post-translational modifications (e.g., oxidation, phosphorylation, and acetylation) that impair the PKM2 tetrameric-mediated metabolic function favoring the PKM2 dimer formation (15, 16). Particularly, an increase in the PKM2 dimer content induces an accumulation of the glycolytic intermediates, including that of G6P, that results in increased metabolic flux into the PPP and enhanced NADPH production (17–20).

6-Phosphogluconate dehydrogenase, the third enzyme involved in PPP, that catalyzes the oxidative decarboxylation of 6-phosphogluconate to ribulose 5-phosphate and CO_2, with concomitant reduction of NADP to NADPH, has also been reported to be upregulated in many solid cancers and has been often correlated to G6PDH (4).

Transketolase and TALDO are the two key enzymes in the non-oxidative branch and divert glycolytic intermediates (e.g., F6P and G3P) into the PPP to fuel ribonucleotides biosynthesis, essential for fast proliferating cancer cells (21). Their expression levels have been found deregulated in cancer cells. Particularly, oncogenes activation that leads to hyper-proliferation may also have an impact on the non-oxidative branch of the PPP, favoring ribonucleotides biosynthesis. Indeed, the use of isogenic colorectal cancer cell lines that express either the mutant or the wild-type form of K-RAS or B-RAF shows that the enzymes of the non-oxidative branch of the PPP (ribose-5-phosphate isomerase and TKT) are upregulated in the K-RAS-mutant cell lines (20). These results are in line with a previous report showing that K-RAS mutations in pancreatic cancers enhance the non-oxidative branch of the PPP (22).

Due to the important role in maintaining the redox homeostasis, PPP is also importantly regulated by nuclear factor erythroid 2-related factor 2 (NRF2), a transcription factor that has an essential role in combating enhanced ROS levels and controlling ROS detoxification and homeostasis. NRF2, which is usually bound in its inactive form to the cytosolic Kelch-like ECH-associated protein 1 (KEAP1), is able to translocate into the nucleus upon NRF2-KEAP1 destabilization, oxidative stress, or protein succination driven by fumarate accumulation (23, 24). Once into the nucleus, NRF2 activates antioxidant response genes and this activation have been reported to play a major role in protecting cancer cells from the oxidative stress induced by antitumoral therapies. Activation of NRF2 or destabilization of NRF2–KEAP1 interaction caused by genetic modifications of NRF2 and KEAP1 has been reported in several cancer types, including those of liver, esophagus, intestine, lung, and breast (25, 26). Moreover, the increased expression of NRF2-dependent genes correlates with poor prognosis (27–29). NRF2 is tightly linked to metabolic reprogramming. In particular, NRF2 targeting impairs the activity and the expression of the enzymes involved in the PPP (e.g., G6PDH, TKT, and 6PGDH), a process that has been shown to be mediated by microRNAs (30). Recently, it has been shown that NRF2 enhances expression of the PPP enzymes. The consequent increase of the PPP flux leads to acquired proliferative advantage in the presence of constitutive activation of PI3K-AKT signaling pathway due to *PTEN* deletion (31). Oncogenic activation of K-RAS and B-RAF and overexpression of MYC, together with enhanced activation of the PI3K/AKT pathway induce NRF2 nuclear translocation, further reinforcing the link between oncogenes activation and NRF2-mediated PPP induction and redox homeostasis alterations.

An important driver of cancer cell growth and survival is the activation of the PI3K/AKT pathway that can occur as a consequence of external stimuli, e.g., growth factor receptor activation or as a consequence of a genetic lesion, e.g., *via* PTEN loss-of-function that ultimately leads to PI3K/AKT-mediated mTOR activation. It has been extensively reported that mTOR can control cell metabolism, including the glycolytic pathway and PPP (32). mTOR kinase can associate with different subunits leading to two signaling complexes, mTORC1 and mTORC2. Particularly, mTORC1 regulates cell proliferation and anabolism.

Indeed, it has been shown that mTORC1 activation sustains the metabolic flux through both glycolysis and the oxidative arm of PPP. Duvel and coworkers demonstrated that mTORC1 activation promotes G6PDH expression, a process that is in part mediated by SREBP1 (33). Additionally, it has been recently reported that mTOR-dependent G6PDH expression can be controlled by androgen receptor signaling in prostate cancer models (34). In the Duvel et al. manuscript it was also reported that mTOR activation induces HIF-1α expression and stabilization, leading to the transcriptional activation of a plethora of metabolic HIF-dependent genes in a hypoxia-independent mechanism (33). HIF-1α stabilization have been also reported to control the expression of TKT in pancreatic cancer cells, hence impacting on the non-oxidative branch of the PPP (35) and subsequent response and resistance to gemcitabine. However, also hypoxia has been reported to impact on PPP flux. Indeed, colon cancer cells subjected to 1% hypoxia increase their intracellular levels of ribose-phosphates and gluconic-acid, terminal and intermediate compounds of the oxidative branch of PPP, respectively (36).

ONE-CARBON METABOLISM (1-CM): THE SERINE SYNTHESIS PATHWAY (SSP)

One-carbon (1-C) metabolism is responsible for the transfer of 1-C unit through folate intermediates, coupling the folate and the methionine cycle. This pathway is required for nucleic acid synthesis (purine and thymidine), amino acids homeostasis (methionine, serine, and glycine), antioxidant defense (NADPH production) and epigenetic maintenance (homocysteine re-methylation) (37–39). In particular, the methionine cycle produces the substrate for the S-adenosyl methionine (SAM)-dependent methyl transferases. These enzymes are responsible for the addition of methyl groups to proteins, lipids, secondary metabolites, and nucleic acids and are, therefore, essential for epigenetic modifications.

In order to sustain the NADPH-mediated antioxidant defense, 1-CM supplements the major source of intracellular NADPH, which is produced by the oxidative branch of the PPP and by the malic enzyme. In the 1-C cycle, there are two steps that can lead to NADPH production: one catalyzed by the methyl dehydrogenases, mitochondrial 2-like (MTHFD2L) and cytosol MTHFD1, and the other catalyzed by the formyl dehydrogenases, cytosolic aldehyde dehydrogenase 1 family member L1 (ALDH1L1) and mitochondrial aldehyde dehydrogenase 1 family member L2 (ALDH1L2).

1-C units are mainly derived from serine, formate, and histidine, which are directly used in the cytosolic folate cycle or by glycine, sarcosine, and dimethylglycine, which are converted into folate and secreted into the cytosol (40, 41). Glycine has a prominent role because it can contribute to both the folate cycle and to serine production. In particular, the folate cycle is completed in the cytosol by serine hydroxymethyltransferase 2 (SHMT2), which catalyzes the concomitant conversion of L-serine to glycine and tetrahydrofolate to 5,10-methyleneTHF. Hence, glycine represents a precursor of glutathione and purines, required for antioxidant defense and proliferation, respectively. If necessary, glycine can be converted into serine with the concomitant production of 5,10-methylene-THF, although some of the specificity of serine-related metabolic functions are lost and this results, for instance, in decreased purine synthesis and reduced cell proliferation (42, 43).

The main donor of 1-C to the folate cycle is the non-essential amino acid serine that can be synthetized *de novo* by the cell (41), a process that requires three MYC-regulated enzymes, PHGDH, PSAT1, and PSPH. The backbone of serine is derived from the glycolytic or gluconeogenic pathway, with the production of the intermediate 3PG. The main transcriptional activator of the three SSP enzymes is ATF4, a cAMP-response element-mediated transcription factor that mediates oxidative stress response (*via* NRF2) (44), serine starvation (*via* mouse double minute 2 homolog MDM2) (45), and histone methylation (41).

At physiological levels, serine can act as an allosteric activator of the metabolic activity of PKM2, which catalyzes the last step of glycolysis, preventing the redirection of 3PG into serine synthesis and promoting glycolysis. Similarly, AKT-mediated phosphorylation of MDM2, a negative regulator of p53, on Ser166 induces the association of MDM2 to PKM2, in order to promote PKM2 activity. In condition of oxidative stress, serine deprivation, and the consequent decrease in the allosteric activation of PKM2, the non-phosphorylated form of MDM2 is recruited to the chromatin with ATF4, which activates a transcriptional program involved in amino acid metabolism and redox homeostasis (45–48). In particular, during oxidative stress, ATF4 has been reported to be transcriptionally activated by NRF2, which induces the transcription of SSP-related genes and regulates the antioxidant response (44). While during serine starvation, the inhibition of PKM2 metabolic activity leads to the accumulation of glycolytic intermediates that results in *de novo* serine synthesis activation, if G3P is used as a substrate for the PHGDH, or into oxidative PPP activation and/or if G6P is used as a substrate for G6PDH, as described above (27).

mTORC1 signaling, through ATF4, activates the transcription of MTHFD2 in both normal and cancer cells, increasing *de novo* purine synthesis necessary for nucleic acid production (48). PKM2-expressing cells can maintain mTORC1 activity and proliferate in serine-depleted medium (49).

It has been reported that highly proliferative cells require an exogenous amount of serine supply for their optimal growth to adjunct the *de novo* synthesis (50). Indeed, additional serine sources are derived from diet intake, from breakdown of intracellular proteins, and from the conversion of glycine. In serine starve condition, P53 null cells have an impaired proliferation rate and tumor growth is reduced in mice fed with serine-free diet (50).

Due to its role in maintaining amino acid and redox homeostasis and epigenetic regulation, it is not surprising that the SSP has been found altered in cancer. Since serine can become a limiting factor, cancer cells can adopt two strategies to bypass this issue: they can increase *de novo* serine synthesis to become dependent on some alternative amino acids, such as glutamine, or can promote exogenous serine intake (41). For instance, PHGDH is commonly altered in cancer cells, either upon gene amplification (51, 52), or activation of transcription factors promoting expression of SSP enzymes, e.g., MYC and NFR2 (44). Among the signaling pathways that promote the 1-C metabolism, it has been reported that HER2 amplification (53) increases the expression of PHGDH (**Figure 1**). The PHGDH promoter is positively regulated by specificity protein 1 (SP1) and nuclear transcription factor Y (NFY), two transcription factors that are often upregulated in cancer (54). PHGDH is overexpressed and associated with poor prognosis in triple negative breast cancer and melanoma (44, 52, 55). While genomic alterations are typical of PHGDH, the high expression of the folate enzyme is due to aberrant transcriptional regulation. In non-small cell lung cancer (NSCLC), ATF4 overexpression can be caused by KEAP1-mediated activation of NRF2 (44) or activation of the PI3K/mTOR signaling pathway (48, 56, 57).

Under hypoxia, HIF-1α and MYC can mediate SHMT2 increased levels that correlate with poor prognosis. Among the enzymes that catalyze the reactions coupled with the production of NADPH, THF dehydrogenases (cytosolic ALDH1L1 and mitochondrial ALDH1L2) and MTHF-dehydrogenases (mitochondria MTHFD2L and cytosolic MTHFD1) are involved in cancer. ALDH1L1 is usually underexpressed in cancer cells (57), as its overexpression would deplete the cytosolic 10-formyl-THF used for the synthesis of nucleotides. Conversely, ALDH1L2 is overexpressed to control mitochondrial redox homeostasis under hypoxia and to support melanoma cells metastatization (56). K-RAS activating mutation is associated with the high expression of the folate metabolism enzyme, MTHFD2 (47), and this leads to increased nucleotide synthesis, increased ATP and NADH production, and finally to enhanced mitochondrial NADPH production which is essential for struggling ROS increased levels. Conversely, during nutrient deprivation or hypoxia, when intracellular levels of ATP decline, 5′ AMP activated kinase (AMPK) activation represses the expression of MTHFD2 (50, 58).

MITOCHONDRIAL METABOLISM

Reactive oxygen species mtROS are generated by mitochondria as a natural by-product of electron transport chain activity. Recently, it has been reported that mitochondria ROS (mtROS) can activate tumorigenic signaling and metabolic reprogramming. Increased production of ROS has long been observed to be a hallmark of many tumors and cancer cell lines. Consequently, this oncogenic signaling increases the expression of antioxidant proteins to balance the high production of ROS to maintain redox homeostasis.

Indeed, several aspects of mitochondrial biology, including biogenesis and turnover, fission and fusion, and mitochondria-controlled metabolic signaling are controlling cellular transformation (59, 60).

Mitochondria are signaling hubs and bioenergetics organelles, which play an important role in cellular adaptation to environmental changes, directly responding to nutrient availability. For instance, during stress condition, such as extracellular acidosis or hypoxia (60, 61), mitochondria tune their metabolism to support fatty acid synthesis by upregulating the reductive carboxylation of α-ketoglutarate *via* citrate generation to support both production of lipid membranes and production of intermediates for protein acetylation (13).

An element controlling mitochondrial function is the mitochondrial mass, which can vary greatly in cell, depending on the microenvironmental context and oxidative fuels availability of certain nutrients. The mitochondrial mass is regulated by biogenesis and turnover, two different pathways that can act as positive and negative regulators of tumorigenesis (59). It is generally accepted that MYC is one of the key activator of mitochondrial biogenesis in cancer, and opposed effects are exerted by the activation of HIF-1α signaling pathway (62), as well as FOXO3a (62–64). In physiological conditions, MYC couples mitochondrial biogenesis with cell-cycle progression, whereas once deregulated in cancer it stimulates mitochondrial metabolism to support rapid

cell growth (63). The major impact of MYC on mitochondria function depends on the direct regulation of the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PPARGC1A, best known as PGC-1α) (58), that is responsible for the enhanced metabolic plasticity of aggressive cancer cells. In particular, PGC-1α levels have a dichotomous effect on tumors, with an upregulation that has been associated with pro- or anti-tumorigenic effects on the basis of tumor types and experimental conditions (65–67).

The discovery of the tumor suppressor liver kinase B1 (LKB1) and its major downstream effectors PGC-1α and AMPK established a central metabolic hub at the crossroad between energy regulation and cancer development (68). It has been already reported that LKB1 plays an important role in reducing intracellular ROS in response to oxidative stress (69, 70). Consequently, LKB1 loss increases oxidative DNA damage and mutations induced by the accumulation of ROS. In NSCLC, the redox imbalance caused by LKB1 inactivation modulates tumor plasticity and promotes tumor progression *via* metabolic adaptation (71).

In addition to aerobic glycolysis, cancer cells often rely on elevated glutaminolysis, supporting mitochondrial metabolism for cancer growth. In order to fuel the tricarboxcylic acid (TCA) cycle, glutamine is first converted to glutamate by glutaminase and then to α-ketoglutarate by glutamate dehydrogenase or aminotransferases. Therefore, glutamine supports the high proliferating rate of cancer cells by acting as substrate for the TCA cycle to produce building blocks, including ATP, lipids, nucleotides, and proteins. MYC oncogene transcriptionally promotes glutaminolysis and the use of glutamine as a bioenergetic substrate (72). The final effect of MYC-dependent glutaminolysis is a profound mitochondrial metabolism reprogramming due to glutamine catabolism dependency and TCA cycle anaplerosis. Noteworthy, reductive carboxylation under hypoxia has been shown to promote generation of 2-hydroxyglutarate (2HG) even in absence of isocitrate dehydrogenase (IDH) mutations (73, 74).

Recently, it has been reported that an association with the oncometabolite 2HG accumulations and MYC pathway activation in breast cancer patients (75). This analysis identified a subtype of tumors characterized by higher levels of 2HG that associate with poor prognosis and with a distinct DNA methylation pattern. These tumors tend to overexpress glutaminase, suggesting a functional relationship between MYC activation and glutamine dependence in breast cancer (75, 76).

2-Hydroxyglutarate is a TCA byproduct that can be generated in certain conditions and has been termed oncometabolite, since its production can drive oncogenesis (77). To date, various germline and somatic mutations in mitochondrial enzymes led to oncometabolites accumulation. For instance, succinate dehydrogenase (SDH), fumarate hydratase, and IDH (78) once mutated lead to cellular transformation and oncogenesis (79) *via* augmented levels of fumarate, succinate, and 2HG, respectively (79). Those molecules present various activity, altering epigenetic pattern, enzymatic activity, or the aforementioned protein succination induced by fumarate. Coherently with the oncogenic role of such metabolite, TRAP1 expression has been shown to induce cellular transformation by inhibiting SDH, thus promoting succinate build-up (80, 81).

Recently, Morita et al. reported a key role for mTORC1 signaling, stimulating mitochondrial biogenesis and activity, bolstering ATP production capacity (82). This effect is mediated by the activity of 4E-BP proteins that mediate mTORC1-driven translation of mitochondria-related mRNAs, mitochondrial respiration and biogenesis, and ATP production. These data reveal a feed-forward mechanism by which translation impacts mitochondrial function to maintain cellular energy homeostasis (82). Consistently, in muose Embryonic Stem cells has been shown that tumorigenicity and teratoma formation was bound to increased mitochondrial metabolism linked to higher mTOR activity (83).

Mitochondria are extremely dynamic structures and the balance of fission and fusion is responsible for their morphology (84). The mitochondrial division is controlled by dynamin-related protein 1 (Drp1), a GTPase that is recruited to the outer mitochondria membrane. Drp1 mitochondrial translocation is regulated by different kinases that are activated by specific cell-cycle and stress conditions. Several studies described that cancer cells have an imbalance fission-fusion state, characterized by high fission and decreased fusion activities, causing a fragmentation of mitochondrial network (85). Disruption of mitochondrial dynamics are one of the key features of K-RAS-induced cellular transformation, by stimulating mitochondrial fragmentation *via* ERK1/2-mediated phosphorylation of Drp1 (86). In addition, the mitochondrial remodeling caused by oncogenic K-RAS directly increases ROS generation and impacts the mitochondrial function itself (87). Similarly to what has been reported for the MYC oncogenic activation, the mitochondrial network remodeling caused by oncogenic K-RAS generates general tumorigenic stimuli that promote transformation. However, further studies are needed to understand the effects of oncogenic signaling pathways on mitochondrial dynamics. K-RAS-mediated metabolic remodeling drives also an augmented glutamine metabolism and malic enzyme activity, acting as an important source of NADPH (**Figure 2**) (88). Mitochondrial remodeling is also altered by ATPase inhibitory factor 1 (ATPIF), a factor exerting pro-tumorigenic function by preventing cristae remodeling and mitochondrial depolarization by inhibiting ATPase activity of complex V (89).

Redox homeostasis is an important process that can fuel anchorage-independent growth. In this stressful condition, mitochondrial IDH2 supports NADPH pool to decrease mtROS accumulation (41). Consequently, the limitation of excessive generation of mtROS is a converging pattern that can support metastasis formation, although a moderate degree of mtROS directly promotes metastasis formation (56, 90). Accordingly, inhibiting mitophagy by BNip3, hence degradation of dysfunctional mitochondria, promotes mtROS generation sufficient to induce metastasis in breast cancer (91).

Another important player in the regulation of mitochondrial metabolism and intracellular redox homeostasis is p53, a key regulator of several biological processes, including energy homeostasis, apoptosis, and cell-cycle arrest, through transcription-dependent and -independent mechanisms (92). To regulate energy metabolism, p53 represses glycolysis, by regulating the

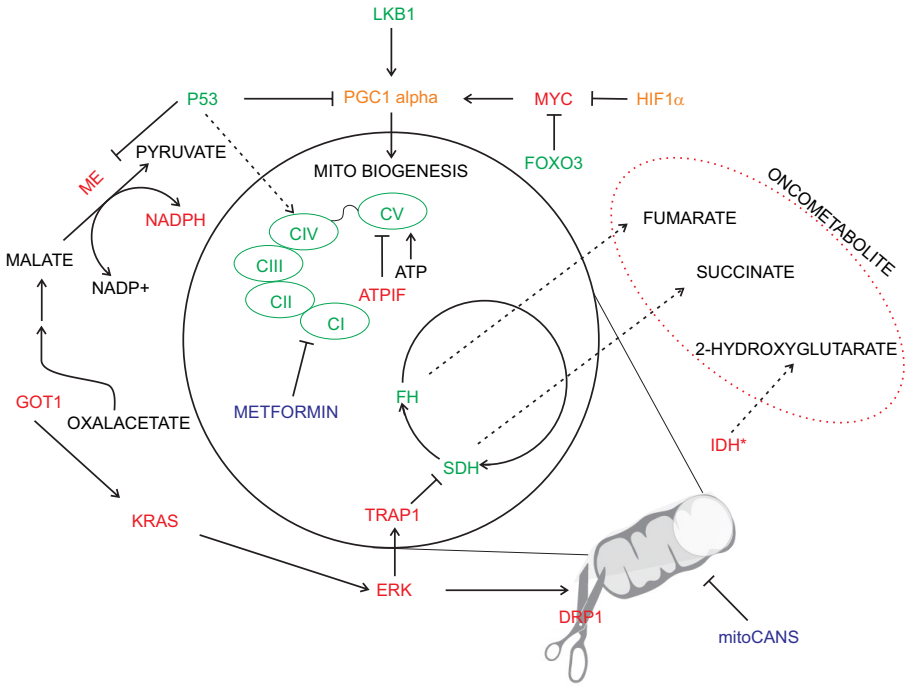


FIGURE 2 | Oncogenic deregulation in mitochondrial metabolism. Physiological mitochondrial metabolism is deranged in cancer by several oncogenic signaling (depicted in red). Inhibition of specific mitochondrial enzymes [i.e., succinate dehydrogenase (SDH) and fumarate hydratase (FH)] or specific mutation [i.e., isocitrate dehydrogenase (IDH)] leads to cellular transformation *via* the generation of oncometabolites. Similarly, oncogenic drivers affect electron transport chain activity and promote increased oxidative stress buffering (e.g., *via* malic enzyme-ME). Pro-tumorigenic signals are represented in red and anti-tumorigenic in green. Enzymes with a double pro/anti-tumorigenic signal according to the type of tumor (i.e., PGC1 alpha and HIF1α) are represented in orange. The main chemotherapy agents are represented in blue.

expression of TIGAR, PGAM1, and PDK2 (pyruvate dehydrogenase kinase 2), and promotes oxidative phosphorylation process.

Beside on its nuclear role for gene expression, a transcription-independent role of p53 in inducing mitochondrial apoptosis has been suggested (93). The mitochondrial regulation of p53 is critical for the control of the cellular switch between survival and apopotosis. In fact, several studies have shown that after DNA damage, p53 is translocated to mitochondrial outer membrane, causing mitochondrial outer membrane permeabilization (MOMP), caspase-3 activation, and cytochrome c release (94).

Altogether these studies demonstrate that mitochondria are crucial players of tumorigenesis, given that this process requires the ability to adapt to cellular and environmental alterations. Therefore, a deep understanding of molecular mechanisms regulating the function of mitochondria will be critical for the next generation of anti-cancer agents.

TARGETING ONCOGENE-DEPENDENT METABOLISM: THERAPEUTIC OPPORTUNITIES

One of the biggest challenges in cancer management is to ensure a tailored therapeutic approach to the patients that will lead to the administration to the right drug (as either monotherapy or combination), at the right patient in the right moment. Target therapies have expanded the portfolio of therapeutic opportunities and are often used either alone or as adjuvant therapies with chemotherapeutic agents. Cancer patients are usually matched to targeted therapies depending on driver mutations profiled in their tumors. However, the metabolic landscape of the tumor profoundly affects the response to therapy (95) and should be considered for optimal therapeutic intervention. Additionally, independently of the oncogenic drivers that sustain cancer growth and progression, understanding the metabolic network and the requirements of a given cancer, will offer a series of additional potential targets that could be exploited therapeutically.

For instance, since the PPP plays a key role in ribonucleotides biosynthesis, necessary for duplicating tumor cells, and redox homeostasis, essential to handle the oxidative stress induced by anti-tumoral therapies, targeting key step of the PPP has been proposed as a potential therapeutic opportunity. However, few inhibitors targeting PPP enzymes are available and no clinical trials have been designed to investigate PPP targeting in solid cancers. However, in the preclinical setting PPP targeting has been effective. Due to the rate-limiting role of the G6PDH enzyme, its targeting has been proposed and a series of compounds able to inhibit its activity *in vitro* and/or *in vivo* have been reported. Among them, the best characterized is the adrenal hormone dehydroepiandrosterone (DHEA), shown to be effective in inhibiting proliferation and survival

of different cancer models (96, 97). To date, 60 trials are (or have been) investigating the application of DHEA in different cancer types (clinicaltrials.gov). However, the majority of the studies have evaluated DHEA for its hormonal effect rather than for its ability to target PPP. In the concluded trials, DHEA is overall well tolerated and further studies should be designed to draw any conclusion on its anti-metabolic effect; accordingly, patients should be subgrouped based on their metabolic dependency, with a particular focus on G6PDH expression. Other compounds have been reported to target G6PDH, such as epigallocatechin gallate (78), chelerythrine (initially a PKC inhibitor), Amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d]pyrimidine (PP2) (98), and the Bay 11–7082 (99) but these compounds will require additional characterization in relevant cancer models prior to enter into the clinical setting. Interestingly, other compounds have been investigated in high-throughput screening leading to additional promising G6PDH inhibitors (100). The PPP can also be blocked by the 6-aminonicotinamide (6-AN) that promotes the biosynthesis of the 6-AN adenine dinucleotide phosphate which is able to inhibit the 6PGDH enzyme, hence reducing the NADPH production (4). In summary, targeting the PPP pathway may be a successful approach because, in addition to the reduction of the ribonucleotide biosynthesis that ultimately impacts on cell division, it could potentiate the effect of other therapies by reducing the resistance to the oxidative stress induced by anti-cancer compounds.

Importantly, for an emerging group of therapies, Mayers and colleagues have proposed that the metabolism of a target tumor may be a key determinant of its response and may be determined by the tumor's tissue of origin and not exclusively by their mutation status [see the Perspective by Vousden and Yang (101)]. Indeed, branched-chain amino acids (BCAAs) destiny has been traced in lung and pancreatic cancer mouse models driven by the same mutations. Lung tumors showed increased BCAA nitrogen uptake and utilization for the biosynthesis of amino acid and nucleotides, while pancreatic tumors displayed decreased uptake of free BCAAs (102). These differences demonstrate that the tissue of origin shapes tumor metabolism and should be taken into account to ensure the optimal therapeutic approach.

It is established that p53 loss is a frequent aberration in many cancer cells and that one of the pro-tumoral effects of such genetic lesion is the increased serine dependency, indicating that limiting cellular serine availability may be a therapeutic option. Tumors with amplified SSP genes are only partially dependent on exogenous serine depletion and a potential therapeutic approach for this subset of cancer may come from impairing the *de novo* serine synthesis. For example, PHGDH inhibitors (21, 51, 103) could be useful in patients with PHGDH-amplified tumors (21, 51). Indeed, chemotherapy based on the inhibition of the folate metabolism and thymidylate synthesis has been the pioneer anti-cancer drugs that entered into the clinical management of cancer patients (104). For instance, methotrexate and 5-fluorouracil are still used in leukemias treatment, while pemetrexed, which acts by mimicking the folic acid and inhibiting the purines and pyrimidines biosynthesis, is administered to patients with mesothelioma and lung cancer (105, 106). Combination of a serine-free diet with metformin, an anti-diabetic biguanide that has been reported to target complex I of the electron transport chain among other targets, shows a synergistic antineoplastic effect (107). Side effects due to the inhibition of 1-CM in non-transformed cells mainly affect proliferating tissues, such as the intestinal epithelium and bone marrow, resulting in gastric damage, anemia, and immune deficiency. Circulating and intratumoral levels of serine and/or the expression of genes of the folate metabolism could be used as potential predictive biomarkers to identify subsets of cancers more likely to respond to anti-folate therapy (47, 108).

Mitochondria are gatekeepers of cell death and could, therefore, be considered as valuable targets for novel therapeutic avenues. The characteristic redox state of malignant cells, due to the altered electrochemical gradient across the inner mitochondrial membrane, renders mitochondria optimal target as shown by Leanza and coworkers (109). Metformin, as described above, has been recently proposed to be repurposed for cancer treatment in light of its activity as mitochondrial complex I inhibitor, promoting cancer specific cell death in a plethora of conditions, most notably in cancer stem cells (110, 111). In cancer tissues, the maintenance of mitochondrial structural integrity is essential to produce energy and overcome cellular stress, including nutrient deprivation and chemotherapy (112). Coherently, major efforts are ongoing for the development of novel molecules targeting directly targeting mitochondria in cancer (i.e., mitoCANS) (113).

CONCLUDING REMARKS

Genomic aberrations play a major role in tumor development and targeting oncogenic drivers have been successful in many types of cancers. However, the advantages of the oncogenic activation in terms of enhancing proliferation and survival capacity, metastatic abilities and resistance to therapies have a profound effect on the metabolic network of a cancer. It is conceivable that, to prolong the efficacy of the current anti-tumoral therapeutic regimens or to combat the development of therapy resistance, a potential successful approach could be the targeting of the "engine" of the tumor growth, i.e., targeting its driving metabolism. Indeed, independently of the drivers that promote tumor progression and/or therapy resistance, metabolic reprogramming will occur, a process that has been proposed to converge according to the tissue of origin more than of the oncogenic drivers (114). Here, we have listed a series of metabolic pathways that are controlled by key oncogenes and oncosuppressors that are often deregulated in cancer and could be potentially targeted. The concomitant targeting of the drivers together with the engine (that sustain cancer growth and progression) will render the signaling and metabolic reprogramming more difficult to occur and could represent a valid therapeutic approach. Further preclinical investigations on synthetic lethality approach in different tumor types and on the potential side effects that such stringent combinatory approach could have on normal cells are required and,

if successful, will represent a major contribution toward the introduction of the personalized therapy approach in cancer patients' management.

AUTHOR CONTRIBUTIONS

MS, PP, MM, and AM selected the references and cowrote the text.

FUNDING

PP is supported by Italian Ministry for University and Research (MIUR, Rita Levi-Montalcini program for young researchers, 2014). MM is funded by Fondazione Umberto Veronesi. AM is funded by Fondazione Umberto Veronesi and Associazione Italiana Ricerca sul Cancro and Fondazione Cassa di Risparmio di Firenze (grant 19515).

REFERENCES

1. Vander Heiden MG, DeBerardinis RJ. Understanding the intersections between metabolism and cancer biology. *Cell* (2017) 168(4):657–69. doi:10.1016/j.cell.2016.12.039
2. Panieri E, Santoro MM. ROS homeostasis and metabolism: a dangerous liason in cancer cells. *Cell Death Dis* (2016) 7(6):e2253. doi:10.1038/cddis.2016.105
3. Patra KC, Hay N. The pentose phosphate pathway and cancer. *Trends Biochem Sci* (2014) 39(8):347–54. doi:10.1016/j.tibs.2014.06.005
4. Kowalik MA, Columbano A, Perra A. Emerging role of the pentose phosphate pathway in hepatocellular carcinoma. *Front Oncol* (2017) 7:87. doi:10.3389/fonc.2017.00087
5. Dore MP, Davoli A, Longo N, Marras G, Pes GM. Glucose-6-phosphate dehydrogenase deficiency and risk of colorectal cancer in Northern Sardinia: a retrospective observational study. *Medicine (Baltimore)* (2016) 95(44):e5254. doi:10.1097/MD.0000000000005254
6. Luzzatto L, Notaro R. Malaria. Protecting against bad air. *Science* (2001) 293(5529):442–3. doi:10.1126/science.1063292
7. Ho HY, Cheng ML, Chiu DT. Glucose-6-phosphate dehydrogenase – beyond the realm of red cell biology. *Free Radic Res* (2014) 48(9):1028–48. doi:10.3109/10715762.2014.913788
8. Floter J, Kaymak I, Schulze A. Regulation of metabolic activity by p53. *Metabolites* (2017) 7(2):1–18. doi:10.3390/metabo7020021
9. Tarrado-Castellarnau M, de Atauri P, Cascante M. Oncogenic regulation of tumor metabolic reprogramming. *Oncotarget* (2016) 7(38):62726–53. doi:10.18632/oncotarget.10911
10. Schwartzenberg-Bar-Yoseph F, Armoni M, Karnieli E. The tumor suppressor p53 down-regulates glucose transporters GLUT1 and GLUT4 gene expression. *Cancer Res* (2004) 64(7):2627–33. doi:10.1158/0008-5472.CAN-03-0846
11. Hitosugi T, Zhou L, Elf S, Fan J, Kang HB, Seo JH, et al. Phosphoglycerate mutase 1 coordinates glycolysis and biosynthesis to promote tumor growth. *Cancer Cell* (2012) 22(5):585–600. doi:10.1016/j.ccr.2012.09.020
12. Cosentino C, Grieco D, Costanzo V. ATM activates the pentose phosphate pathway promoting anti-oxidant defence and DNA repair. *EMBO J* (2011) 30(3):546–55. doi:10.1038/emboj.2010.330
13. Corbet C, Feron O. Tumour acidosis: from the passenger to the driver's seat. *Nat Rev Cancer* (2017) 17(10):577–93. doi:10.1038/nrc.2017.77
14. Patra KC, Wang Q, Bhaskar PT, Miller L, Wang Z, Wheaton W, et al. Hexokinase 2 is required for tumor initiation and maintenance and its systemic deletion is therapeutic in mouse models of cancer. *Cancer Cell* (2013) 24(2):213–28. doi:10.1016/j.ccr.2013.06.014
15. Giannoni E, Taddei ML, Morandi A, Comito G, Calvani M, Bianchini F, et al. Targeting stromal-induced pyruvate kinase M2 nuclear translocation impairs oxphos and prostate cancer metastatic spread. *Oncotarget* (2015) 6(27):24061–74. doi:10.18632/oncotarget.4448
16. Dayton TL, Jacks T, Vander Heiden MG. PKM2, cancer metabolism, and the road ahead. *EMBO Rep* (2016) 17(12):1721–30. doi:10.15252/embr.201643300
17. Kumar B, Bamezai RN. Moderate DNA damage promotes metabolic flux into PPP via PKM2 Y-105 phosphorylation: a feature that favours cancer cells. *Mol Biol Rep* (2015) 42(8):1317–21. doi:10.1007/s11033-015-3876-8
18. Fukuda S, Miyata H, Miyazaki Y, Makino T, Takahashi T, Kurokawa Y, et al. Pyruvate kinase M2 modulates esophageal squamous cell carcinoma chemotherapy response by regulating the pentose phosphate pathway. *Ann Surg Oncol* (2015) 22(Suppl 3):S1461–8. doi:10.1245/s10434-015-4522-3
19. Cui Y, Nadiminty N, Liu C, Lou W, Schwartz CT, Gao AC. Upregulation of glucose metabolism by NF-kappaB2/p52 mediates enzalutamide resistance in castration-resistant prostate cancer cells. *Endocr Relat Cancer* (2014) 21(3):435–42. doi:10.1530/ERC-14-0107
20. Hutton JE, Wang X, Zimmerman LJ, Slebos RJ, Trenary IA, Young JD, et al. Oncogenic KRAS and BRAF drive metabolic reprogramming in colorectal cancer. *Mol Cell Proteomics* (2016) 15(9):2924–38. doi:10.1074/mcp.M116.058925
21. Pacold E, Brimacombe KR, Chan SH, Rohde JM, Lewis CA, Swier LJ, et al. A PHGDH inhibitor reveals coordination of serine synthesis and one-carbon unit fate. *Nat Chem Biol* (2016) 12(6):452–8. doi:10.1038/nchembio.2070
22. Ying H, Kimmelman AC, Lyssiotis CA, Hua S, Chu GC, Fletcher-Sananikone E, et al. Oncogenic KRAS maintains pancreatic tumors through regulation of anabolic glucose metabolism. *Cell* (2012) 149(3):656–70. doi:10.1016/j.cell.2012.01.058
23. Dodson M, Redmann M, Rajasekaran NS, Darley-Usmar V, Zhang J. KEAP1-NRF2 signalling and autophagy in protection against oxidative and reductive proteotoxicity. *Biochem J* (2015) 469(3):347–55. doi:10.1042/BJ20150568
24. Kinch L, Grishin NV, Brugarolas J. Succination of Keap1 and activation of Nrf2-dependent antioxidant pathways in FH-deficient papillary renal cell carcinoma type 2. *Cancer Cell* (2011) 20(4):418–20. doi:10.1016/j.ccr.2011.10.005
25. Menegon S, Columbano A, Giordano S. The dual roles of NRF2 in cancer. *Trends Mol Med* (2016) 22(7):578–93. doi:10.1016/j.molmed.2016.05.002
26. Jaramillo MC, Zhang DD. The emerging role of the Nrf2-Keap1 signaling pathway in cancer. *Genes Dev* (2013) 27(20):2179–91. doi:10.1101/gad.225680.113
27. Chaneton B, Hillmann P, Zheng L, Martin ACL, Maddocks ODK, Chokkathukalam A, et al. Serine is a natural ligand and allosteric activator of pyruvate kinase M2. *Nature* (2012) 491(7424):458–62. doi:10.1038/nature11540
28. Solis LM, Behrens C, Dong W, Suraokar M, Ozburn NC, Moran CA, et al. Nrf2 and Keap1 abnormalities in non-small cell lung carcinoma and association with clinicopathologic features. *Clin Cancer Res* (2010) 16(14):3743–53. doi:10.1158/1078-0432.CCR-09-3352
29. Shibata T, Ohta T, Tong KI, Kokubu A, Odogawa R, Tsuta K, et al. Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy. *Proc Natl Acad Sci U S A* (2008) 105(36):13568–73. doi:10.1073/pnas.0806268105
30. Singh A, Happel C, Manna SK, Acquaah-Mensah G, Carrerero J, Kumar S, et al. Transcription factor NRF2 regulates miR-1 and miR-206 to drive tumorigenesis. *J Clin Invest* (2013) 123(7):2921–34. doi:10.1172/JCI66353
31. Mitsuishi Y, Taguchi K, Kawatani Y, Shibata T, Nukiwa T, Aburatani H, et al. Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. *Cancer Cell* (2012) 22(1):66–79. doi:10.1016/j.ccr.2012.05.016
32. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell* (2012) 149(2):274–93. doi:10.1016/j.cell.2012.03.017
33. Duvel K, Yecies JL, Menon S, Raman P, Lipovsky AI, Souza AL, et al. Activation of a metabolic gene regulatory network downstream of mTOR complex 1. *Mol Cell* (2010) 39(2):171–83. doi:10.1016/j.molcel.2010.06.022
34. Tsouko E, Khan AS, White MA, Han JJ, Shi Y, Merchant FA, et al. Regulation of the pentose phosphate pathway by an androgen receptor-mTOR-mediated mechanism and its role in prostate cancer cell growth. *Oncogenesis* (2014) 3:e103. doi:10.1038/oncsis.2014.18

35. Shukla SK, Purohit V, Mehla K, Gunda V, Chaika NV, Vernucci E, et al. MUC1 and HIF-1alpha signaling crosstalk induces anabolic glucose metabolism to impart gemcitabine resistance to pancreatic cancer. *Cancer Cell* (2017) 32(3):392. doi:10.1016/j.ccell.2017.06.004
36. Frezza C, Zheng L, Tennant DA, Papkovsky DB, Hedley BA, Kalna G, et al. Metabolic profiling of hypoxic cells revealed a catabolic signature required for cell survival. *PLoS One* (2011) 6(9):e24411. doi:10.1371/journal.pone.0024411
37. Amelio I, Cutruzzola F, Antonov A, Agostini M, Melino G. Serine and glycine metabolism in cancer. *Trends Biochem Sci* (2014) 39(4):191–8. doi:10.1016/j.tibs.2014.02.004
38. Mattaini KR, Sullivan MR, Vander Heiden MG. The importance of serine metabolism in cancer. *J Cell Biol* (2016) 214(3):249–57. doi:10.1083/jcb.201604085
39. Yang M, Vousden KH. Serine and one-carbon metabolism in cancer. *Nat Rev Cancer* (2016) 16(10):650–62. doi:10.1038/nrc.2016.81
40. Ducker GS, Rabinowitz JD. One-carbon metabolism in health and disease. *Cell Metab* (2017) 25(1):27–42. doi:10.1016/j.cmet.2016.08.009
41. Jiang L, Shestov AA, Swain P, Yang C, Parker SJ, Wang QA, et al. Reductive carboxylation supports redox homeostasis during anchorage-independent growth. *Nature* (2016) 532(7598):255–8. doi:10.1038/nature17393
42. Jain M, Nilsson R, Sharma S, Madhusudhan N, Kitami T, Souza AL, et al. Metabolite profiling identifies a key role for glycine in rapid cancer cell proliferation. *Science* (2012) 336(6084):1040–4. doi:10.1126/science.1218595
43. Newman AC, Maddocks ODK. One-carbon metabolism in cancer. *Br J Cancer* (2017) 116(12):1499–504. doi:10.1038/bjc.2017.118
44. DeNicola GM, Chen PH, Mullarky E, Sudderth JA, Hu Z, Wu D, et al. NRF2 regulates serine biosynthesis in non-small cell lung cancer. *Nat Genet* (2015) 47(12):1475–81. doi:10.1038/ng.3421
45. Riscal R, Schrepfer E, Arena G, Cisse MY, Bellvert F, Heuillet M, et al. Chromatin-bound MDM2 regulates serine metabolism and redox homeostasis independently of p53. *Mol Cell* (2016) 62(6):890–902. doi:10.1016/j.molcel.2016.04.033
46. Mayo LD, Donner DB. A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *Proc Natl Acad Sci U S A* (2001) 98(20):11598–603. doi:10.1073/pnas.181181198
47. Moran DM, Trusk PB, Pry K, Paz K, Sidransky D, Bacus SS. KRAS mutation status is associated with enhanced dependency on folate metabolism pathways in non-small cell lung cancer cells. *Mol Cancer Ther* (2014) 13(6):1611–24. doi:10.1158/1535-7163.MCT-13-0649
48. Ben-Sahra I, Hoxhaj G, Ricoult SJH, Asara JM, Manning BD. mTORC1 induces purine synthesis through control of the mitochondrial tetrahydrofolate cycle. *Science* (2016) 351(6274):728–33. doi:10.1126/science.aad0489
49. Ye J, Mancuso A, Tong X, Ward PS, Fan J, Rabinowitz JD, et al. Pyruvate kinase M2 promotes de novo serine synthesis to sustain mTORC1 activity and cell proliferation. *Proc Natl Acad Sci U S A* (2012) 109(18):6904–9. doi:10.1073/pnas.1204176109
50. Maddocks OD, Berkers CR, Mason SM, Zheng L, Blyth K, Gottlieb E, et al. Serine starvation induces stress and p53-dependent metabolic remodelling in cancer cells. *Nature* (2013) 493(7433):542–6. doi:10.1038/nature11743
51. Mullarky E, Lucki NC, Beheshti Zavareh R, Anglin JL, Gomes AP, Nicolay BN, et al. Identification of a small molecule inhibitor of 3-phosphoglycerate dehydrogenase to target serine biosynthesis in cancers. *Proc Natl Acad Sci U S A* (2016) 113(7):1778–83. doi:10.1073/pnas.1521548113
52. Locasale JW, Grassian AR, Melman T, Lyssiotis CA, Mattaini KR, Bass AJ, et al. Phosphoglycerate dehydrogenase diverts glycolytic flux and contributes to oncogenesis. *Nat Genet* (2011) 43(9):869–74. doi:10.1038/ng.890
53. Bollig-Fischer A, Dewey TG, Ethier SP. Oncogene activation induces metabolic transformation resulting in insulin-independence in human breast cancer cells. *PLoS One* (2011) 6(3):e17959. doi:10.1371/journal.pone.0017959
54. Jun DY, Park HS, Lee JY, Baek JY, Park HK, Fukui K, et al. Positive regulation of promoter activity of human 3-phosphoglycerate dehydrogenase (PHGDH) gene is mediated by transcription factors Sp1 and NF-Y. *Gene* (2008) 414(1–2):106–14. doi:10.1016/j.gene.2008.02.018
55. Possemato R, Marks KM, Shaul YD, Pacold ME, Kim D, Birsoy K, et al. Functional genomics reveal that the serine synthesis pathway is essential in breast cancer. *Nature* (2011) 476(7360):346–50. doi:10.1038/nature10350
56. Piskounova E, Agathocleous M, Murphy MM, Hu Z, Huddlestun SE, Zhao Z, et al. Oxidative stress inhibits distant metastasis by human melanoma cells. *Nature* (2015) 527(7577):186–91. doi:10.1038/nature15726
57. Anguera MC, Field MS, Perry C, Ghandour H, Chiang EP, Selhub J, et al. Regulation of folate-mediated one-carbon metabolism by 10-formyltetrahydrofolate dehydrogenase. *J Biol Chem* (2006) 281(27):18335–42. doi:10.1074/jbc.M510623200
58. Audet-Walsh E, Papadopoli DJ, Gravel SP, Yee T, Bridon G, Caron M, et al. The PGC-1alpha/ERRalpha axis represses one-carbon metabolism and promotes sensitivity to anti-folate therapy in breast cancer. *Cell Rep* (2016) 14(4):920–31. doi:10.1016/j.celrep.2015.12.086
59. Vyas S, Zaganjor E, Haigis MC. Mitochondria and cancer. *Cell* (2016) 166(3):555–66. doi:10.1016/j.cell.2016.07.002
60. Porporato PE, Filigheddu N, Bravo-San Pedro JM, Kroemer G, Galluzzi L. Mitochondrial metabolism and cancer. *Cell Res* (2017) 3:265–80. doi:10.1038/cr.2017.155
61. Corbet C, Pinto A, Martherus R, Santiago de Jesus JP, Polet F, Feron O. Acidosis drives the reprogramming of fatty acid metabolism in cancer cells through changes in mitochondrial and histone acetylation. *Cell Metab* (2016) 24(2):311–23. doi:10.1016/j.cmet.2016.07.003
62. Dang CV. MYC, metabolism, cell growth, and tumorigenesis. *Cold Spring Harb Perspect Med* (2013) 3(8):1–15. doi:10.1101/cshperspect.a014217
63. Stine ZE, Walton ZE, Altman BJ, Hsieh AL, Dang CV. MYC, metabolism, and cancer. *Cancer Discov* (2015) 5(10):1024–39. doi:10.1158/2159-8290.CD-15-0507
64. Ferber EC, Peck B, Delpuech O, Bell GP, East P, Schulze A. FOXO3a regulates reactive oxygen metabolism by inhibiting mitochondrial gene expression. *Cell Death Differ* (2012) 19(6):968–79. doi:10.1038/cdd.2011.179
65. Torrano V, Valcarcel-Jimenez L, Cortazar AR, Liu X, Urosevic J, Castillo-Martin M, et al. The metabolic co-regulator PGC1alpha suppresses prostate cancer metastasis. *Nat Cell Biol* (2016) 18(6):645–56. doi:10.1038/ncb3357
66. Andrzejewski S, Klimcakova E, Johnson RM, Tabaries S, Annis MG, McGuirk S, et al. PGC-1alpha promotes breast cancer metastasis and confers bioenergetic flexibility against metabolic drugs. *Cell Metab* (2017) 26(5):778–87.e5. doi:10.1016/j.cmet.2017.09.006
67. LeBleu VS, O'Connell JT, Gonzalez Herrera KN, Wikman H, Pantel K, Haigis MC, et al. PGC-1alpha mediates mitochondrial biogenesis and oxidative phosphorylation in cancer cells to promote metastasis. *Nat Cell Biol* (2014) 16:10. doi:10.1038/ncb3039
68. Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, DePinho RA, et al. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc Natl Acad Sci U S A* (2004) 101(10):3329–35. doi:10.1073/pnas.0308061100
69. Jimenez AI, Fernandez P, Dominguez O, Dopazo A, Sanchez-Cespedes M. Growth and molecular profile of lung cancer cells expressing ectopic LKB1: down-regulation of the phosphatidylinositol 3'-phosphate kinase/PTEN pathway. *Cancer Res* (2003) 63(6):1382–8.
70. Xu HG, Zhai YX, Chen J, Lu Y, Wang JW, Quan CS, et al. LKB1 reduces ROS-mediated cell damage via activation of p38. *Oncogene* (2015) 34(29):3848–59. doi:10.1038/onc.2014.315
71. Li F, Han X, Wang R, Wang H, Gao Y, Wang X, et al. LKB1 Inactivation elicits a redox imbalance to modulate non-small cell lung cancer plasticity and therapeutic response. *Cancer Cell* (2015) 27(5):698–711. doi:10.1016/j.ccell.2015.04.001
72. Pavlova NN, Thompson CB. The emerging hallmarks of cancer metabolism. *Cell Metab* (2016) 23(1):27–47. doi:10.1016/j.cmet.2015.12.006
73. Wise DR, Ward PS, Shay JE, Cross JR, Gruber JJ, Sachdeva UM, et al. Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of alpha-ketoglutarate to citrate to support cell growth and viability. *Proc Natl Acad Sci U S A* (2011) 108(49):19611–6. doi:10.1073/pnas.1117773108
74. Jiang L, Boufersaoui A, Yang C, Ko B, Rakheja D, Guevara G, et al. Quantitative metabolic flux analysis reveals an unconventional pathway of fatty acid synthesis in cancer cells deficient for the mitochondrial citrate

transport protein. *Metab Eng* (2017) 43(Pt B):198–207. doi:10.1016/j.ymben.2016.11.004
75. Terunuma A, Putluri N, Mishra P, Mathe EA, Dorsey TH, Yi M, et al. MYC-driven accumulation of 2-hydroxyglutarate is associated with breast cancer prognosis. *J Clin Invest* (2014) 124(1):398–412. doi:10.1172/JCI71180
76. Mishra P, Tang W, Putluri V, Dorsey TH, Jin F, Wang F, et al. ADHFE1 is a breast cancer oncogene and induces metabolic reprogramming. *J Clin Invest* (2018) 128(1):323–40. doi:10.1172/JCI93815
77. Frezza C. Mitochondrial metabolites: undercover signalling molecules. *Interface Focus* (2017) 7(2):20160100. doi:10.1098/rsfs.2016.0100
78. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, et al. An integrated genomic analysis of human glioblastoma multiforme. *Science* (2008) 321(5897):1807–12. doi:10.1126/science.1164382
79. Sciacovelli M, Frezza C. Oncometabolites: unconventional triggers of oncogenic signalling cascades. *Free Radic Biol Med* (2016) 100:175–81. doi:10.1016/j.freeradbiomed.2016.04.025
80. Sciacovelli M, Guzzo G, Morello V, Frezza C, Zheng L, Nannini N, et al. The mitochondrial chaperone TRAP1 promotes neoplastic growth by inhibiting succinate dehydrogenase. *Cell Metab* (2013) 17(6):988–99. doi:10.1016/j.cmet.2013.04.019
81. Masgras I, Ciscato F, Brunati AM, Tibaldi E, Indraccolo S, Curtarello M, et al. Absence of neurofibromin induces an oncogenic metabolic switch via mitochondrial ERK-mediated phosphorylation of the chaperone TRAP1. *Cell Rep* (2017) 18(3):659–72. doi:10.1016/j.celrep.2016.12.056
82. Morita M, Gravel SP, Chenard V, Sikstrom K, Zheng L, Alain T, et al. mTORC1 controls mitochondrial activity and biogenesis through 4E-BP-dependent translational regulation. *Cell Metab* (2013) 18(5):698–711. doi:10.1016/j.cmet.2013.10.001
83. Schieke SM, Ma M, Cao L, McCoy JP Jr, Liu C, Hensel NF, et al. Mitochondrial metabolism modulates differentiation and teratoma formation capacity in mouse embryonic stem cells. *J Biol Chem* (2008) 283(42):28506–12. doi:10.1074/jbc.M802763200
84. Mishra P, Chan DC. Metabolic regulation of mitochondrial dynamics. *J Cell Biol* (2016) 212(4):379–87. doi:10.1083/jcb.201511036
85. Senft D, Ronai ZA. Adaptive stress responses during tumor metastasis and dormancy. *Trends Cancer* (2016) 2(8):429–42. doi:10.1016/j.trecan.2016.06.004
86. Kashatus JA, Nascimento A, Myers LJ, Sher A, Byrne FL, Hoehn KL, et al. Erk2 phosphorylation of Drp1 promotes mitochondrial fission and MAPK-driven tumor growth. *Mol Cell* (2015) 57(3):537–51. doi:10.1016/j.molcel.2015.01.002
87. Serasinghe MN, Wieder SY, Renault TT, Elkholi R, Asciolla JJ, Yao JL, et al. Mitochondrial division is requisite to RAS-induced transformation and targeted by oncogenic MAPK pathway inhibitors. *Mol Cell* (2015) 57(3):521–36. doi:10.1016/j.molcel.2015.01.003
88. Son J, Lyssiotis CA, Ying H, Wang X, Hua S, Ligorio M, et al. Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway. *Nature* (2013) 496(7443):101–5. doi:10.1038/nature12040
89. Faccenda D, Nakamura J, Gorini G, Dhoot GK, Piacentini M, Yoshida M, et al. Control of mitochondrial remodeling by the ATPase inhibitory factor 1 unveils a pro-survival relay via OPA1. *Cell Rep* (2017) 18(8):1869–83. doi:10.1016/j.celrep.2017.01.070
90. Porporato PE, Payen VL, Perez-Escuredo J, De Saedeleer CJ, Danhier P, Copetti T, et al. A mitochondrial switch promotes tumor metastasis. *Cell Rep* (2014) 8(3):754–66. doi:10.1016/j.celrep.2014.06.043
91. Chourasia AH, Tracy K, Frankenberger C, Boland ML, Sharifi MN, Drake LE, et al. Mitophagy defects arising from BNip3 loss promote mammary tumor progression to metastasis. *EMBO Rep* (2015) 16(9):1145–63. doi:10.15252/embr.201540759
92. Liu J, Zhang C, Hu W, Feng Z. Tumor suppressor p53 and its mutants in cancer metabolism. *Cancer Lett* (2015) 356(2 Pt A):197–203. doi:10.1016/j.canlet.2013.12.025
93. Vaseva AV, Marchenko ND, Moll UM. The transcription-independent mitochondrial p53 program is a major contributor to nutlin-induced apoptosis in tumor cells. *Cell Cycle* (2009) 8(11):1711–9. doi:10.4161/cc.8.11.8596
94. Vaseva AV, Moll UM. The mitochondrial p53 pathway. *Biochim Biophys Acta* (2009) 1787(5):414–20. doi:10.1016/j.bbabio.2008.10.005
95. Morandi A, Indraccolo S. Linking metabolic reprogramming to therapy resistance in cancer. *Biochim Biophys Acta* (2017) 1868(1):1–6. doi:10.1016/j.bbcan.2016.12.004
96. Kowalik MA, Guzzo G, Morandi A, Perra A, Menegon S, Masgras I, et al. Metabolic reprogramming identifies the most aggressive lesions at early phases of hepatic carcinogenesis. *Oncotarget* (2016) 7(22):32375–93. doi:10.18632/oncotarget.8632
97. Ramos-Montoya A, Lee WN, Bassilian S, Lim S, Trebukhina RV, Kazhyna MV, et al. Pentose phosphate cycle oxidative and nonoxidative balance: a new vulnerable target for overcoming drug resistance in cancer. *Int J Cancer* (2006) 119(12):2733–41. doi:10.1002/ijc.22227
98. Gupte RS, Vijay V, Marks B, Levine RJ, Sabbah HN, Wolin MS, et al. Upregulation of glucose-6-phosphate dehydrogenase and NAD(P)H oxidase activity increases oxidative stress in failing human heart. *J Card Fail* (2007) 13(6):497–506. doi:10.1016/j.cardfail.2007.04.003
99. Ghashghaeinia M, Giustarini D, Koralkova P, Koberle M, Alzoubi K, Bissinger R, et al. Pharmacological targeting of glucose-6-phosphate dehydrogenase in human erythrocytes by Bay 11-7082, parthenolide and dimethyl fumarate. *Sci Rep* (2016) 6:28754. doi:10.1038/srep28754
100. Preuss J, Richardson AD, Pinkerton A, Hedrick M, Sergienko E, Rahlfs S, et al. Identification and characterization of novel human glucose-6-phosphate dehydrogenase inhibitors. *J Biomol Screen* (2013) 18(3):286–97. doi:10.1177/1087057112462131
101. Vousden KH, Yang M. Location, location, location. *Science* (2016) 353(6304):1095–6. doi:10.1126/science.aai7629
102. Mayers JR, Torrence ME, Danai LV, Papagiannakopoulos T, Davidson SM, Bauer MR, et al. Tissue of origin dictates branched-chain amino acid metabolism in mutant KRAS-driven cancers. *Science* (2016) 353(6304):1161–5. doi:10.1126/science.aaf5171
103. Wang Q, Liberti MV, Liu P, Deng X, Liu Y, Locasale JW, et al. Rational design of selective allosteric inhibitors of PHGDH and serine synthesis with anti-tumor activity. *Cell Chem Biol* (2017) 24(1):55–65. doi:10.1016/j.chembiol.2016.11.013
104. Locasale JW. Serine, glycine and one-carbon units: cancer metabolism in full circle. *Nat Rev Cancer* (2013) 13(8):572–83. doi:10.1038/nrc3557
105. Ciuleanu T, Brodowicz T, Zielinski C, Kim JH, Krzakowski M, Laack E, et al. Maintenance pemetrexed plus best supportive care versus placebo plus best supportive care for non-small-cell lung cancer: a randomised, double-blind, phase 3 study. *Lancet* (2009) 374(9699):1432–40. doi:10.1016/S0140-6736(09)61497-5
106. Zalcman G, Mazieres J, Margery J, Greillier L, Audigier-Valette C, Moro-Sibilot D, et al. Bevacizumab for newly diagnosed pleural mesothelioma in the mesothelioma avastin cisplatin pemetrexed study (MAPS): a randomised, controlled, open-label, phase 3 trial. *Lancet* (2016) 387(10026):1405–14. doi:10.1016/S0140-6736(15)01238-6
107. Gravel SP, Hulea L, Toban N, Birman E, Blouin MJ, Zakikhani M, et al. Serine deprivation enhances antineoplastic activity of biguanides. *Cancer Res* (2014) 74(24):7521–33. doi:10.1158/0008-5472.CAN-14-2643-T
108. Valik D, Radina M, Sterba J, Vojtesek B. Homocysteine: exploring its potential as a pharmacodynamic biomarker of antifolate chemotherapy. *Pharmacogenomics* (2004) 5(8):1151–62. doi:10.1517/14622416.5.8.1151
109. Leanza L, Romio M, Becker KA, Azzolini M, Trentin L, Manago A, et al. Direct pharmacological targeting of a mitochondrial ion channel selectively kills tumor cells in vivo. *Cancer Cell* (2017) 31(4):516–531e10. doi:10.1016/j.ccell.2017.03.003
110. Sancho P, Burgos-Ramos E, Tavera A, Bou Kheir T, Jagust P, Schoenhals M, et al. MYC/PGC-1alpha balance determines the metabolic phenotype and plasticity of pancreatic cancer stem cells. *Cell Metab* (2015) 22(4):590–605. doi:10.1016/j.cmet.2015.08.015
111. Danhier P, Banski P, Payen VL, Grasso D, Ippolito L, Sonveaux P, et al. Cancer metabolism in space and time: beyond the Warburg effect. *Biochim Biophys Acta* (2017) 1858(8):556–72. doi:10.1016/j.bbabio.2017.02.001
112. Wheaton WW, Weinberg SE, Hamanaka RB, Soberanes S, Sullivan LB, Anso E, et al. Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis. *Elife* (2014) 3:e02242. doi:10.7554/eLife.02242
113. Neuzil J, Dong LF, Rohlena J, Truksa J, Ralph SJ. Classification of mitocans, anti-cancer drugs acting on mitochondria. *Mitochondrion* (2013) 13(3):199–208. doi:10.1016/j.mito.2012.07.112

114. Gaude E, Frezza C. Tissue-specific and convergent metabolic transformation of cancer correlates with metastatic potential and patient survival. *Nat Commun* (2016) 7:13041. doi:10.1038/ncomms13041

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

MINI REVIEW
published: 07 March 2018
doi: 10.3389/fonc.2018.00053

The Role of Mitochondrial H+-ATP Synthase in Cancer

*Pau B. Esparza-Moltó and José M. Cuezva**

Departamento de Biología Molecular, Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Centro de Investigación Biomédica en Red de Enfermedades Raras CIBERER-ISCIII, Instituto de Investigación Hospital 12 de Octubre (i+12), Universidad Autónoma de Madrid, Madrid, Spain

Cancer cells reprogram energy metabolism by boosting aerobic glycolysis as a main pathway for the provision of metabolic energy and of precursors for anabolic purposes. Accordingly, the relative expression of the catalytic subunit of the mitochondrial H+-ATP synthase—the core hub of oxidative phosphorylation—is downregulated in human carcinomas when compared with its expression in normal tissues. Moreover, some prevalent carcinomas also upregulate the ATPase inhibitory factor 1 (IF1), which is the physiological inhibitor of the H+-ATP synthase. IF1 overexpression, both in cells in culture and in tissue-specific mouse models, is sufficient to reprogram energy metabolism to an enhanced glycolysis by limiting ATP production by the H+-ATP synthase. Furthermore, the IF1-mediated inhibition of the H+-ATP synthase promotes the production of mitochondrial ROS (mtROS). mtROS modulate signaling pathways favoring cellular proliferation and invasion, the activation of antioxidant defenses, resistance to cell death, and modulation of the tissue immune response, favoring the acquisition of several cancer traits. Consistently, IF1 expression is an independent marker of cancer prognosis. By contrast, inhibition of the H+-ATP synthase by α-ketoglutarate and the oncometabolite 2-hydroxyglutarate, reduces mTOR signaling, suppresses cancer cell growth, and contributes to lifespan extension in several model organisms. Hence, the H+-ATP synthase appears as a conserved hub in mitochondria-to-nucleus signaling controlling cell fate. Unraveling the molecular mechanisms responsible for IF1 upregulation in cancer and the signaling cascades that are modulated by the H+-ATP synthase are of utmost interest to decipher the metabolic and redox circuits contributing to cancer origin and progression.

Keywords: oxidative phosphorylation, ATPase inhibitory factor 1, mitohormesis, metabolic reprogramming, hepatocarcinogenesis, inflammation

OPEN ACCESS

Edited by:
Andrea Rasola, Università degli Studi di Padova, Italy

Reviewed by:
Giovanna Lippe, University of Udine, Italy
Valentina Giorgio, Institute of Neuroscience (CNR), Italy

***Correspondence:**
José M. Cuezva
jmcuezva@cbm.csic.es

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *15 January 2018*
Accepted: *20 February 2018*
Published: *07 March 2018*

Citation:
Esparza-Moltó PB and Cuezva JM (2018) The Role of Mitochondrial H+-ATP Synthase in Cancer. Front. Oncol. 8:53. doi: 10.3389/fonc.2018.00053

OVERVIEW OF METABOLIC REPROGRAMMING IN CANCER

Cancer cells experience a series of alterations during oncogenic transformation that confer them new features (1). Cellular metabolism is a central player in the acquisition of this new phenotype. Indeed, cancer cells are highly proliferative and readapt their metabolism to meet the demands imposed by the new phenotype, namely higher requirements of metabolic energy and of precursors for biosynthetic purposes (**Figure 1**) (2–5). One prominent feature of the metabolic reprogramming experienced by cancer cells is an enhanced glycolytic rate in the presence of oxygen, what is known as aerobic glycolysis (6) (**Figure 1**). Glycolysis provides cancer cells with various metabolic precursors that serve for the synthesis of amino acids, nucleotides, and lipids, as well as reducing power and ATP. In addition, mitochondrial function is readapted during oncogenic

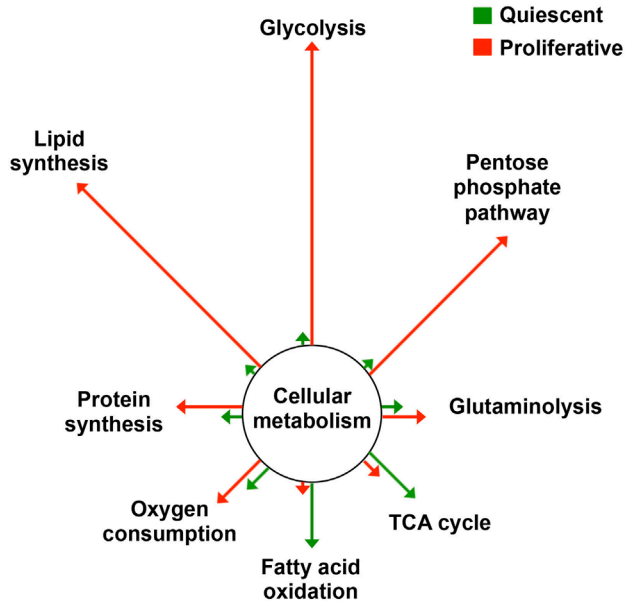


FIGURE 1 | Metabolic reprogramming in proliferation. The changes in flux through the main pathways of cellular metabolism are depicted in proliferative cells (red) relative to that of quiescent cells (green). Arrows' length is proportional to the relative change in flux. Data adapted from Ref. (2–5). Abbreviation: TCA cycle, tricarboxylic acid cycle.

transformation and mutations in genes encoding mitochondrial proteins contribute to cancer development (7, 8). In particular, the relative contribution of mitochondria to energy provision is reduced, the organelles becoming mostly dedicated to produce anabolic precursors through the tricarboxylic acid (TCA) cycle (9) (**Figure 1**). Likewise, mitochondria, which are crucial hubs in intracellular signaling (10), readapt this function in cancer (11). Intermediates of the TCA cycle contribute to signaling tumorigenesis (12) and mtROS, which are key mediators in mitochondrial communication (13), activate signaling pathways that promote cell proliferation and tumorigenesis (14, 15). Moreover, mitochondrial dynamics is also reprogrammed in cancer (16).

THE H^+-ATP SYNTHASE IS DOWNREGULATED IN CANCER

A critical enzyme complex within the mitochondria is the H^+-ATP synthase, the rotatory engine of the inner mitochondrial membrane responsible for ATP synthesis by oxidative phosphorylation (OXPHOS) (17). The H^+-ATP synthase consumes the proton electrochemical gradient generated across the inner mitochondrial membrane by the electron transport chain to drive ATP synthesis (17, 18). In addition, the H^+-ATP synthase is a critical component of the mitochondrial permeability transition pore (PTP) whose prolonged opening triggers the execution of cell death (19–21). Although its mechanism of participation in PTP opening is currently debated (22–24), recent findings have mapped the residues in subunits of the H^+-ATP synthase for Ca^{2+} activation (25) and pH inhibition (26) of the PTP, reinforcing the role of the H^+-ATP synthase in PTP function and providing first evidence that single point mutations in the enzyme affect PTP modulation. Hence, the H^+-ATP synthase integrates the bioenergetic and death-signaling functions of mitochondria, what makes it a relevant target for oncogenic transformation (27, 28). Actually, mutations in the mitochondrial-encoded subunit *a* of the H^+-ATP synthase (MT-ATP6), which are found in different human carcinomas, promote tumor growth by restraining cell death (29, 30). Recent findings in yeast MT-ATP6 mutants have confirmed the role of these mutations in the PTP response to Ca^{2+} (31), providing additional genetic evidence that supports the involvement of mutations in the H^+-ATP synthase in PTP functioning during carcinogenesis. However, it should be noted that the two mutations in MT-ATP6 impact the PTP response only when the function of the outer mitochondrial membrane porin complex is perturbed (i.e., OM45-GFP background) (31). In fact, permeability transition has been documented in rho0 cells that lack mtDNA (32), highlighting the relevance of the genetic background of the cancer cell for the desensitization of the PTP.

Regardless of oncogenic mutations on the H^+-ATP synthase, it has been documented that the relative expression of the catalytic subunit of the complex (β-F1-ATPase) is downregulated in most prevalent human carcinomas when compared with the corresponding normal tissues (33, 34) [for review, see Ref. (2)]. The relative expression of β-F1-ATPase in the tissue provides a "bioenergetic signature" of the carcinoma that informs of the overall capacity of mitochondria. The bioenergetic signature [also known as the bioenergetic cellular (BEC) index (2)], is assessed as the protein ratio between β-F1-ATPase and GAPDH and has been shown to be significantly reduced in colon, lung, breast, gastric, and renal carcinomas (2, 33). Interestingly, the quantification of these two proteins in carcinomas derived from different tissues (lung, esophagus, and breast) show similar quantities irrespective of the large differences found in their content in normal tissues (35). These findings support that during oncogenic transformation the tissue-specific differences in energy metabolism are abolished to converge on a similar phenotype to support tumor growth (35). In addition, the BEC index is a biomarker for cancer prognosis and response to therapy. In fact, a higher BEC index predicts a better overall survival and/or disease-free survival in acute myeloid leukemia patients and in colon, lung, breast, and ovarian cancer patients (36–42). These findings thus support that an impaired bioenergetic function of mitochondria favors recurrence and progression of the disease. Moreover, the BEC index also provides a tool for predicting the therapeutic response to various chemotherapeutic strategies aimed at combating tumor progression (43–46).

From a mechanistic view point the control of β-F1-ATPase expression is essentially exerted at post-transcriptional levels (47). In this regard, the translation of β-F1-ATPase mRNA (β-mRNA) both during development and in oncogenesis requires the specific activity of a *cis* element in the 3′ untranslated region of the mRNA that tightly controls its translation by RNA binding proteins (48–53) and miRNAs (54).

THE DIVERSE ROLE OF INHIBITORY FACTOR 1 (IF1) IN HUMAN CARCINOMAS

Besides the lower BEC index found in tumors, some prevalent human carcinomas also upregulate the expression of the ATPase IF1, which is the physiological inhibitor of the H^+-ATP synthase (55, 56). Classically, IF1 was thought to function only to prevent mitochondrial ATP consumption by the reverse activity of the H^+-ATP synthase (ATP hydrolase), which happens when mitochondria become de-energized such as in ischemia or in hypoxia (57, 58). However, more recent findings indicate that IF1 can bind to the H^+-ATP synthase under normal phosphorylating conditions, hence inhibiting also the forward ATP synthetic activity of the enzyme (59). It should be noted that when arguing about the inhibition exerted by IF1 on the H^+-ATP synthase it is important to take into consideration the tissue content of IF1 and the molar ratio that exists between IF1 and the H^+-ATP synthase because the tissue availability of the inhibitor affects, among other factors, its interaction with the enzyme by the mass–action ratio. Unfortunately, the information of the tissue content of these two proteins in human and mouse tissues is presently missing.

In addition, it should be stressed that IF1 binding to the H^+-ATP synthase, and hence its activity as an inhibitor of the enzyme, is subjected to a stringent posttranslational regulation of the protein by phosphorylation (59). In this regard, we have shown that IF1 is phosphorylated in S39 by a mitochondrial cAMP-dependent protein kinase that renders IF1 unable to bind to the H^+-ATP synthase and hence inactive as an inhibitor of the enzyme (59). Regulation of IF1 phosphorylation depends on the cellular metabolic state to allow the fine tuning of ATP production to the cellular metabolic demand (56, 59). In this regard, we should stress that IF1 is found dephosphorylated, and hence active as an inhibitor of the enzyme in colon, lung, and breast carcinomas as well as in hypoxic cells and in cells progressing through the S/G2/M phases of the cell cycle (59).

In addition, IF1 is sharply upregulated in colon, lung, breast, and ovarian carcinomas, which are tissues that under normal physiological conditions are essentially devoid of the inhibitory protein (60–62). Not surprisingly, IF1 is an independent prognostic marker of disease progression for patients bearing these carcinomas. In non-small cell lung cancer (63), bladder carcinomas (64), and gliomas (65), a high expression level of IF1 in the tumor predicts a worse patient prognosis. On the contrary, in colon and breast cancer patients, a high level of IF1 expression predicts a better outcome (60, 66), especially in the bad prognosis group of triple-negative breast cancer patients (67). In the case of breast cancer, lymph node metastases show a lower expression level of IF1 when compared with the primary tumors (68). This finding suggested that breast cancer cells expressing low levels of IF1 may have a higher metastatic potential, which is in full agreement with our recent finding that low IF1 expression in triple-negative breast cancer cells confers a more invasive phenotype (67).

By contrast, human tissues that express high levels of IF1 under basal physiological conditions such as endometrium, kidney, liver, and stomach do not experience a relevant increase in IF1 expression by oncogenesis (60, 62). Nevertheless, in hepatocarcinomas (69) and in gastric carcinomas (70), a higher tumor content of IF1 predicts a worse prognosis for the patients. However, it should be mentioned that a higher IF1-mRNA expression level is correlated with a better prognosis in patients bearing the intestinal subtype of gastric cancer (66). This apparent discrepancy might arise from the histological type of gastric carcinomas analyzed in both studies and/or because the expression of IF1 in human carcinomas is primarily exerted at posttranscriptional levels (60). Overall, these findings support that IF1 plays a relevant role in cancer origin and progression. However, it remains to be elucidated the differential role played by IF1 in favoring or repressing cancer progression in different types of carcinomas, strongly emphasizing the need for specific studies in cellular, xenograft, and genetically modified mouse models in which to address these issues (67, 71).

THE IMPACT OF IF1-MEDIATED INHIBITION OF THE H^+-ATP SYNTHASE IN CANCER

The role of the H^+-ATP synthase in cancer and in signaling has been studied by developing cellular and mouse models with regulated expression of IF1. The overexpression of IF1 both in cultured cells (55, 60, 61, 67) and in different tissues *in vivo* (71–73) is sufficient to promote metabolic reprogramming to an enhanced aerobic glycolysis (**Figure 2**). Upregulation of glycolysis results from the limitation of cellular ATP availability as a result of the inhibition of the H^+-ATP synthase, supporting that the rate of ATP production by OXPHOS defines the rate of glucose consumption by aerobic glycolysis (74, 75). Likewise, this metabolic situation triggers the activation of the energy sensor AMPK (71–73) (**Figure 2**). Conversely, the silencing of IF1 in cells that express high levels of the protein has the opposite metabolic effect (55, 60, 61).

The inhibition of the H^+-ATP synthase by IF1 also reduces the backflow of protons into the mitochondrial matrix, triggering mitochondrial hyperpolarization and a mild increase in the production of mitochondrial ROS (mtROS) (**Figure 2**), both *in vitro* and *in vivo* (55, 60, 61, 67, 72, 73). In cells and tissues overexpressing IF1 mtROS trigger the carbonylation of some cellular proteins and signal the activation of the canonical nuclear factor kappa B (NFκB) pathway (71–73) (**Figure 2**). In colon cancer cells, NFκB induces a nuclear transcriptional program that favors cellular proliferation, invasion, and evasion of cell death (61). These results are in line with other findings reporting that mtROS are necessary for proliferation and tumorigenesis and that ROS scavenging with mitochondrial-targeted antioxidants reduces cancer cell growth and prosurvival pathways (14, 76, 77). A recent study also argues that IF1 overexpression in carcinomas might contribute to cancer progression by limiting the processing of the pro-fusion dynamin-related protein optic atrophy 1 and thus limiting cristae remodeling during apoptosis (78).

However, the phenotypic changes triggered by IF1 overexpression in colon cancer cells cannot be generalized to other cellular types. In fact, the transcriptional program triggered by IF1 overexpression in triple-negative breast cancer cells supports just the opposite, a less proliferative and invasive phenotype (67). This phenotype for breast cancer cells was confirmed by functional

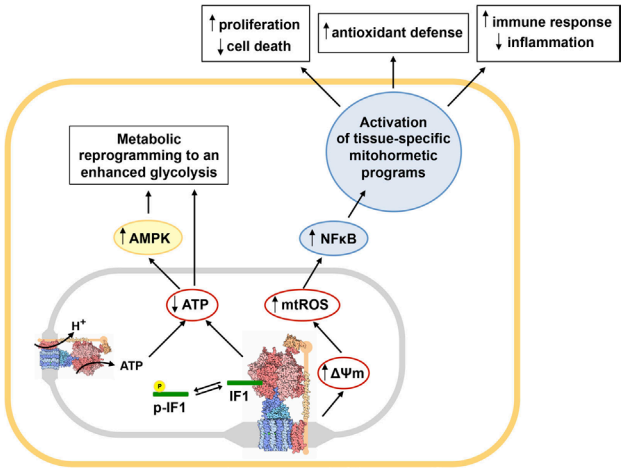


FIGURE 2 | Main metabolic and redox circuits regulated by the inhibition of the H⁺-ATP synthase by inhibitory factor 1 (IF1). Dephosphorylated IF1 (green rod) inhibits the H⁺-ATP synthase (structure) when bound to the enzyme, while IF1 phosphorylation (yellow) prevents its binding and hence its inhibitory activity (59). The inhibition of a relevant pool of molecules of H⁺-ATP synthase by IF1 reduces cellular ATP availability, promoting metabolic reprogramming to an enhanced glycolysis and the activation of AMPK (71–73). Moreover, IF1 inhibition of the H⁺-ATP synthase triggers mitochondrial hyperpolarization because prevents H⁺ backflow through the enzyme enhancing the production of mitochondrial ROS (mtROS) that activate the canonical NFκB pathway (61, 72, 73). Activation of NFκB triggers the induction of different tissue-specific mitohormetic programs in the nucleus of the cell. In colon cancer cells overexpressing IF1, these programs favor proliferation, invasion, and resistance to cell death (61). In transgenic mice overexpressing IF1 in neurons or liver, promote the activation of survival pathways and antioxidant defenses (71, 72). In transgenic mice overexpressing IF1 in the intestine, the activated programs modulate the immune response of the tissue, favoring the development of an anti-inflammatory phenotype (73). Abbreviations: p-IF1, phosphorylated IF1; AMPK, AMP-activated protein kinase; NFκB, nuclear factor kappa B. The structure of the H⁺-ATP synthase was obtained from PDB.

analysis illustrating that IF1 overexpression promotes cell adhesion and maintenance of the extracellular matrix hampering epithelial to mesenchymal transition (67). Accordingly, the results may explain why breast cancer patients with high IF1 expression in the carcinoma have a better prognosis (60, 67).

In the mouse model overexpressing IF1-H49K (a constitutively active mutant of IF1) in the liver, we have shown that downregulation of OXPHOS triggers the induction of AMPK rendering a liver phenotype that is prone to cancer development (71). Indeed, transgenic mice when challenged with the carcinogen diethylnitrosamine develop more and bigger tumors than control mice because there is more extensive proliferation and diminished apoptosis of liver cells (71). Remarkably, IF1 overexpression in human hepatocarcinomas also triggers the activation of NFκB (**Figure 2**), which drives the promotion of angiogenesis and epithelial to mesenchymal transition (69). Not surprisingly, the expression of IF1 in liver cancer predicts a bad overall prognosis and the recurrence of the disease in these patients (69).

Mechanistically, although dimers of H⁺-ATP synthase are critical components of the PTP (19), and the overexpression of IF1 in the liver *in vivo* favors the formation of dimers of the enzyme (71), we have observed that cell death protection is not related to differential opening and regulation of the PTP (71). Actually, we support that the cell death protection afforded by IF1 overexpression in the liver is related to mitohormetic signaling through the induction of an antioxidant response guided by Nrf2 [nuclear factor (erythroid-derived 2)-like 2] (**Figure 2**) (71) because the metabolically preconditioned hepatocytes are more resistant to acetaminophen induced toxicity (71). Interestingly, Nrf2 upregulation is also a strategy deployed by cancer cells to detoxify the higher ROS levels that are produced in these cells (79).

IF1-MEDIATED INHIBITION OF THE H⁺-ATP SYNTHASE MODULATES METABOLIC AND REDOX CIRCUITS

Besides the liver, overexpression of IF1-H49K in neurons *in vivo* also reprograms energy metabolism to an enhanced glycolysis and affords metabolic preconditioning (72). In fact, mice overexpressing IF1-H49K in forebrain neurons are partially protected from excitotoxic damage induced by striatal administration of quinolinic acid because preconditioning partially protects neurons from death, reducing the lesion area in the brain and improving motor performance of the transgenic mice (72). Preconditioning in neurons also involves AMPK activation and mtROS-mediated signaling to implement a Bcl-xL-mediated protection of neurons from apoptosis (72) (**Figure 2**). Other findings also support the neuroprotective role of IF1 in ischemia reoxygenation by promoting autophagy and maintaining mitochondrial bioenergetics (80), although the contribution of IF1 to promotion of autophagy in neurons remains to be studied in the *in vivo* model.

Interestingly, the signaling pathways triggered by the IF1-mediated inhibition of the H⁺-ATP synthase are not limited to the cells overexpressing IF1 but also implicate non-cell autonomous processes. The transgenic mice overexpressing IF1 in enterocytes also show metabolic reprogramming to an enhanced glycolysis and activation of mtROS–NFκB signaling pathway (73) (**Figure 2**). Transgenic mice are partially protected from intestinal inflammation after the administration of the inflammatory agent dextran sodium sulfate (DSS), due to increased recruitment of regulatory T cells and macrophages that are mainly polarized to the M2 phenotype (73). These findings are consistent with the anti-inflammatory phenotype afforded by the overexpression of IF1 as revealed by the higher levels of anti-inflammatory cytokines present in plasma and intestine of the transgenic mice (73). Colonocytes of control mice induce the oncogenic Akt/mTOR/p70S6K and pro-inflammatory STAT3 pathways upon administration of DSS, something that is not observed in IF1-transgenic mice (73). Preconditioning and protection against stress in colon of IF1-transgenic mice clearly responds to the basal activation of the NFκB pathway due to mtROS production (73) (**Figure 2**), because protection from intestinal inflammation is blunted when an mtROS scavenger or an inhibitor of NFκB are administered (73).

Overall, transgenic mice overexpressing IF1 in liver, brain, or intestine reveal that by partial inhibition of OXPHOS and the production of mtROS the tissues acquire an advantageous

phenotype against different forms of oxidative stress and inflammation (71–73) (**Figure 2**), stressing the role of the H^+-ATP synthase as a therapeutic target in diverse human pathologies.

LONGEVITY AND THE INHIBITION OF THE H^+-ATP SYNTHASE

Besides IF1, the H^+-ATP synthase can be inhibited by some mitochondrial metabolites produced in the TCA cycle, such as α-ketoglutarate (α-KG) (81). Therefore, partial inhibition of the enzyme by α-KG also reduces ATP availability and TOR signaling, promoting autophagy in *Caenorhabditis elegans* (81). In addition, the oncometabolite (R)-2-hydroxyglutarate (2-HG), which is structurally similar to α-KG, also inhibits the H^+-ATP synthase both in *C. elegans* and in mammalian cell lines (82). 2-HG is highly accumulated in some gliomas and acute myeloid leukemias that harbor mutations in the genes encoding the cytosolic and mitochondrial isocitrate dehydrogenases (IDH1 and IDH2, respectively). These mutations result in neomorphic enzymes with higher affinity for α-KG that catalyze its conversion into 2-HG (83, 84). Interestingly, the inhibition of the H^+-ATP synthase by 2-HG or α-KG in glioblastoma cells triggers cell growth arrest and cell death under conditions of limited glucose (82). These results are consistent with previous findings that indicate that brain cancer patients with IDH mutations have a longer median overall survival than patients without mutations (83, 85). The suppression of cell growth may be ascribed to reduced ATP levels and mTOR signaling in the tumors (82).

Interestingly, both α-KG and 2-HG, by inhibiting the H^+-ATP synthase, also extend lifespan in *C. elegans* (81, 82), and α-KG might play a role in longevity induced by dietary restriction (81). Other interventions targeting the H^+-ATP synthase have also been shown to extend lifespan in several model organisms, as recently reviewed (62). For instance, silencing of subunits of the H^+-ATP synthase in *C. elegans* and *Drosophila melanogaster* promotes longevity (86, 87). No contribution to longevity has been reported so far in mammals for the modulation of the H^+-ATP synthase. However, recent findings indicate that the specific inhibition of the enzyme by the small molecule J147 prevents the age-associated drift of the hippocampal transcriptome and plasma metabolome in mice and extends lifespan in *D. melanogaster,* providing an additional link between the activity of the H^+-ATP synthase, aging and age-associated pathologies such as dementia (88).

Overall, these findings support the notion that the H^+-ATP synthase is also a conserved hub in intracellular signaling that plays a key role in signaling mitohormesis contributing to cell fate decisions and longevity.

CONCLUDING REMARKS

Reprogramming cellular metabolism is a hallmark of cancer that is necessary to fulfill the metabolic demands imposed by the oncogenic process. In this context, the mitochondrial H^+-ATP synthase is a main hub in rewiring energy metabolism and in retrograde signaling to the nucleus programs required for cancer progression. In this regard, most prevalent carcinomas show reduced expression of the catalytic subunit of the H^+-ATP synthase (β-F1-ATPase) relative to the glycolytic GAPDH, what provides a protein signature of energy metabolism of clinical relevance in oncogenesis. Moreover, some prevalent carcinomas also show an increased expression of IF1, the physiological inhibitor of the enzyme. As revealed in different *in vitro* and *in vivo* systems, IF1 overexpression is sufficient to rewire energy metabolism to an enhanced glycolysis and to trigger an mtROS signal that promotes nuclear reprogramming. IF1-mediated reprogramming is mainly geared by the activation of AMPK and NFκB pathways resulting in the induction of tissue-specific programs aimed at preventing cell death, oxidative damage or inflammation. The precise molecular events that lead to the upregulation of IF1 in cancer and its role in cancer progression in different carcinomas remain to be established. The H^+-ATP synthase, engine of OXPHOS, is also a crucial hub in mitohormetic signaling to modulate cytoprotective defenses that contribute to longevity in several organisms. Therefore, deciphering the metabolic and redox circuits controlled by the H^+-ATP synthase and IF1 are of utmost importance to understand how they contribute to oncogenesis and thus providing new targets for cancer and age-associated diseases.

AUTHOR CONTRIBUTIONS

PE-M and JC wrote the paper. All the authors read, contributed, and approved the final manuscript.

ACKNOWLEDGMENTS

PE-M was supported by a predoctoral fellowship from Fundación La Caixa (Obra Social La Caixa). The work was supported by grants from MINECO (SAF2016-75916-R), CIBERER-ISCIII, and Fundación Ramón Areces, Spain.

REFERENCES

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) 144(5):646–74. doi:10.1016/j.cell.2011.02.013
2. Cuezva JM, Ortega AD, Willers I, Sanchez-Cenizo L, Aldea M, Sanchez-Arago M. The tumor suppressor function of mitochondria: translation into the clinics. *Biochim Biophys Acta* (2009) 1792(12):1145–58. doi:10.1016/j.bbadis.2009.01.006
3. Wang R, Green DR. Metabolic reprogramming and metabolic dependency in Tcells.*ImmunolRev*(2012)249(1):14–26.doi:10.1111/j.1600-065X.2012.01155.x
4. Vander Heiden MG, Lunt SY, Dayton TL, Fiske BP, Israelsen WJ, Mattaini KR, et al. Metabolic pathway alterations that support cell proliferation. *Cold Spring Harb Symp Quant Biol* (2013) 76:325–34. doi:10.1101/sqb.2012.76.010900
5. Lunt SY, Vander Heiden MG. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu Rev Cell Dev Biol* (2011) 27:441–64. doi:10.1146/annurev-cellbio-092910-154237
6. Warburg O. *Metabolism of Tumors*. London: Arnold Constable (1930).
7. Gaude E, Frezza C. Defects in mitochondrial metabolism and cancer. *Cancer Metab* (2014) 2:10. doi:10.1186/2049-3002-2-10

8. Sciacovelli M, Goncalves E, Johnson TI, Zecchini VR, Da Costa AS, Gaude E, et al. Fumarate is an epigenetic modifier that elicits epithelial-to-mesenchymal transition. *Nature* (2016) 537(7621):544–7. doi:10.1038/nature19353
9. Zong WX, Rabinowitz JD, White E. Mitochondria and cancer. *Mol Cell* (2016) 61(5):667–76. doi:10.1016/j.molcel.2016.02.011
10. Chandel NS. Evolution of mitochondria as signaling organelles. *Cell Metab* (2015) 22(2):204–6. doi:10.1016/j.cmet.2015.05.013
11. Sassano ML, Van Vliet AR, Agostinis P. Mitochondria-associated membranes as networking platforms and regulators of cancer cell fate. *Front Oncol* (2017) 7:174. doi:10.3389/fonc.2017.00174
12. Raimundo N, Baysal BE, Shadel GS. Revisiting the TCA cycle: signaling to tumor formation. *Trends Mol Med* (2011) 17(11):641–9. doi:10.1016/j.molmed.2011.06.001
13. Shadel GS, Horvath TL. Mitochondrial ROS signaling in organismal homeostasis. *Cell* (2015) 163(3):560–9. doi:10.1016/j.cell.2015.10.001
14. Weinberg F, Hamanaka R, Wheaton WW, Weinberg S, Joseph J, Lopez M, et al. Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. *Proc Natl Acad Sci U S A* (2010) 107(19):8788–93. doi:10.1073/pnas.1003428107
15. Woo DK, Green PD, Santos JH, D'souza AD, Walther Z, Martin WD, et al. Mitochondrial genome instability and ROS enhance intestinal tumorigenesis in APC(Min/+) mice. *Am J Pathol* (2012) 180(1):24–31. doi:10.1016/j.ajpath.2011.10.003
16. Pendin D, Filadi R, Pizzo P. The concerted action of mitochondrial dynamics and positioning: new characters in cancer onset and progression. *Front Oncol* (2017) 7:102. doi:10.3389/fonc.2017.00102
17. Walker JE. The ATP synthase: the understood, the uncertain and the unknown. *Biochem Soc Trans* (2013) 41(1):1–16. doi:10.1042/BST20110773
18. Saita E, Suzuki T, Kinosita K Jr, Yoshida M. Simple mechanism whereby the F1-ATPase motor rotates with near-perfect chemomechanical energy conversion. *Proc Natl Acad Sci U S A* (2015) 112(31):9626–31. doi:10.1073/pnas.1422885112
19. Giorgio V, Von Stockum S, Antoniel M, Fabbro A, Fogolari F, Forte M, et al. Dimers of mitochondrial ATP synthase form the permeability transition pore. *Proc Natl Acad Sci U S A* (2013) 110(15):5887–92. doi:10.1073/pnas.1217823110
20. Alavian KN, Beutner G, Lazrove E, Sacchetti S, Park HA, Licznerski P, et al. An uncoupling channel within the c-subunit ring of the F1FO ATP synthase is the mitochondrial permeability transition pore. *Proc Natl Acad Sci U S A* (2014) 111(29):10580–5. doi:10.1073/pnas.1401591111
21. Bernardi P, Rasola A, Forte M, Lippe G. The mitochondrial permeability transition pore: channel formation by F-ATP synthase, integration in signal transduction, and role in pathophysiology. *Physiol Rev* (2015) 95(4):1111–55. doi:10.1152/physrev.00001.2015
22. He J, Carroll J, Ding S, Fearnley IM, Walker JE. Permeability transition in human mitochondria persists in the absence of peripheral stalk subunits of ATP synthase. *Proc Natl Acad Sci U S A* (2017) 114(34):9086–91. doi:10.1073/pnas.1711201114
23. He J, Ford HC, Carroll J, Ding S, Fearnley IM, Walker JE. Persistence of the mitochondrial permeability transition in the absence of subunit c of human ATP synthase. *Proc Natl Acad Sci U S A* (2017) 114(13):3409–14. doi:10.1073/pnas.1702357114
24. Giorgio V, Guo L, Bassot C, Petronilli V, Bernardi P. Calcium and regulation of the mitochondrial permeability transition. *Cell Calcium* (2017) 70:56–63. doi:10.1016/j.ceca.2017.05.004
25. Giorgio V, Burchell V, Schiavone M, Bassot C, Minervini G, Petronilli V, et al. Ca(2+) binding to F-ATP synthase beta subunit triggers the mitochondrial permeability transition. *EMBO Rep* (2017) 18(7):1065–76. doi:10.15252/embr.201643354
26. Antoniel M, Jones K, Antonucci S, Spolaore B, Fogolari F, Petronilli V, et al. The unique histidine in OSCP subunit of F-ATP synthase mediates inhibition of the permeability transition pore by acidic pH. *EMBO Rep* (2018) 19(2):257–68. doi:10.15252/embr.201744705
27. Sanchez-Arago M, Formentini L, Cuezva JM. Mitochondria-mediated energy adaption in cancer: the H(+)-ATP synthase-geared switch of metabolism in human tumors. *Antioxid Redox Signal* (2013) 19(3):285–98. doi:10.1089/ars.2012.4883
28. Martinez-Reyes I, Cuezva JM. The H(+)-ATP synthase: a gate to ROS-mediated cell death or cell survival. *Biochim Biophys Acta* (2014) 1837(7):1099–112. doi:10.1016/j.bbabio.2014.03.010
29. Petros JA, Baumann AK, Ruiz-Pesini E, Amin MB, Sun CQ, Hall J, et al. mtDNA mutations increase tumorigenicity in prostate cancer. *Proc Natl Acad Sci U S A* (2005) 102(3):719–24. doi:10.1073/pnas.0408894102
30. Shidara Y, Yamagata K, Kanamori T, Nakano K, Kwong JQ, Manfredi G, et al. Positive contribution of pathogenic mutations in the mitochondrial genome to the promotion of cancer by prevention from apoptosis. *Cancer Res* (2005) 65(5):1655–63. doi:10.1158/0008-5472.CAN-04-2012
31. Niedzwiecka K, Tisi R, Penna S, Lichocka M, Plochocka D, Kucharczyk R. Two mutations in mitochondrial ATP6 gene of ATP synthase, related to human cancer, affect ROS, calcium homeostasis and mitochondrial permeability transition in yeast. *Biochim Biophys Acta* (2018) 1865(1):117–31. doi:10.1016/j.bbamcr.2017.10.003
32. Masgras I, Rasola A, Bernardi P. Induction of the permeability transition pore in cells depleted of mitochondrial DNA. *Biochim Biophys Acta* (2012) 1817(10):1860–6. doi:10.1016/j.bbabio.2012.02.022
33. Cuezva JM, Krajewska M, De Heredia ML, Krajewski S, Santamaria G, Kim H, et al. The bioenergetic signature of cancer: a marker of tumor progression. *Cancer Res* (2002) 62(22):6674–81.
34. Isidoro A, Martinez M, Fernandez PL, Ortega AD, Santamaria G, Chamorro M, et al. Alteration of the bioenergetic phenotype of mitochondria is a hallmark of breast, gastric, lung and oesophageal cancer. *Biochem J* (2004) 378(Pt 1):17–20. doi:10.1042/bj20031541
35. Acebo P, Giner D, Calvo P, Blanco-Rivero A, Ortega AD, Fernandez PL, et al. Cancer abolishes the tissue type-specific differences in the phenotype of energetic metabolism. *Transl Oncol* (2009) 2(3):138–45. doi:10.1593/tlo.09106
36. Cuezva JM, Chen G, Alonso AM, Isidoro A, Misek DE, Hanash SM, et al. The bioenergetic signature of lung adenocarcinomas is a molecular marker of cancer diagnosis and prognosis. *Carcinogenesis* (2004) 25(7):1157–63. doi:10.1093/carcin/bgh113
37. Isidoro A, Casado E, Redondo A, Acebo P, Espinosa E, Alonso AM, et al. Breast carcinomas fulfill the Warburg hypothesis and provide metabolic markers of cancer prognosis. *Carcinogenesis* (2005) 26(12):2095–104. doi:10.1093/carcin/bgi188
38. Lopez-Rios F, Sanchez-Arago M, Garcia-Garcia E, Ortega AD, Berrendero JR, Pozo-Rodriguez F, et al. Loss of the mitochondrial bioenergetic capacity underlies the glucose avidity of carcinomas. *Cancer Res* (2007) 67(19):9013–7. doi:10.1158/0008-5472.CAN-07-1678
39. Lin PC, Lin JK, Yang SH, Wang HS, Li AF, Chang SC. Expression of beta-F1-ATPase and mitochondrial transcription factor A and the change in mitochondrial DNA content in colorectal cancer: clinical data analysis and evidence from an in vitro study. *Int J Colorectal Dis* (2008) 23(12):1223–32. doi:10.1007/s00384-008-0539-4
40. Aldea M, Clofent J, Nunez De Arenas C, Chamorro M, Velasco M, Berrendero JR, et al. Reverse phase protein microarrays quantify and validate the bioenergetic signature as biomarker in colorectal cancer. *Cancer Lett* (2011) 311(2):210–8. doi:10.1016/j.canlet.2011.07.022
41. Hjerpe E, Egyhazi Brage S, Carlson J, Frostvik Stolt M, Schedvins K, Johansson H, et al. Metabolic markers GAPDH, PKM2, ATP5B and BEC-index in advanced serous ovarian cancer. *BMC Clin Pathol* (2013) 13(1):30. doi:10.1186/1472-6890-13-30
42. Xiao X, Yang J, Li R, Liu S, Xu Y, Zheng W, et al. Deregulation of mitochondrial ATPsyn-beta in acute myeloid leukemia cells and with increased drug resistance. *PLoS One* (2013) 8(12):e83610. doi:10.1371/journal.pone.0083610
43. Shin YK, Yoo BC, Chang HJ, Jeon E, Hong SH, Jung MS, et al. Down-regulation of mitochondrial F1F0-ATP synthase in human colon cancer cells with induced 5-fluorouracil resistance. *Cancer Res* (2005) 65(8):3162–70. doi:10.1158/0008-5472.CAN-04-3300
44. Li RJ, Zhang GS, Chen YH, Zhu JF, Lu QJ, Gong FJ, et al. Down-regulation of mitochondrial ATPase by hypermethylation mechanism in chronic myeloid leukemia is associated with multidrug resistance. *Ann Oncol* (2010) 7:1506–14. doi:10.1093/annonc/mdp569
45. Sanchez-Arago M, Cuezva JM. The bioenergetic signature of isogenic colon cancer cells predicts the cell death response to treatment with 3-bromopyruvate, iodoacetate or 5-fluorouracil. *J Transl Med* (2011) 9:19. doi:10.1186/1479-5876-9-19
46. Yizhak K, Le Devedec SE, Rogkoti VM, Baenke F, De Boer VC, Frezza C, et al. A computational study of the Warburg effect identifies metabolic targets inhibiting cancer migration. *Mol Syst Biol* (2014) 10:744. doi:10.15252/msb.20134993

47. Willers IM, Cuezva JM. Post-transcriptional regulation of the mitochondrial H(+)-ATP synthase: a key regulator of the metabolic phenotype in cancer. *Biochim Biophys Acta* (2011) 1807(6):543–51. doi:10.1016/j.bbabio.2010.10.016
48. Izquierdo JM, Cuezva JM. Control of the translational efficiency of beta-F1-ATPase mRNA depends on the regulation of a protein that binds the 3' untranslated region of the mRNA. *Mol Cell Biol* (1997) 17(9):5255–68. doi:10.1128/MCB.17.9.5255
49. Izquierdo JM, Cuezva JM. Internal-ribosome-entry-site functional activity of the 3'-untranslated region of the mRNA for the beta subunit of mitochondrial H+-ATP synthase. *Biochem J* (2000) 346(Pt 3):849–55. doi:10.1042/bj3460849
50. De Heredia ML, Izquierdo JM, Cuezva JM. A conserved mechanism for controlling the translation of beta-F1-ATPase mRNA between the fetal liver and cancer cells. *J Biol Chem* (2000) 275(10):7430–7. doi:10.1074/jbc.275.10.7430
51. Willers IM, Isidoro A, Ortega AD, Fernandez PL, Cuezva JM. Selective inhibition of beta-F1-ATPase mRNA translation in human tumours. *Biochem J* (2010) 426(3):319–26. doi:10.1042/BJ20091570
52. Ortega AD, Sala S, Espinosa E, Gonzalez-Baron M, Cuezva JM. HuR and the bioenergetic signature of breast cancer: a low tumor expression of the RNA-binding protein predicts a higher risk of disease recurrence. *Carcinogenesis* (2008) 29(11):2053–61. doi:10.1093/carcin/bgn185
53. Ortega AD, Willers IM, Sala S, Cuezva JM. Human G3BP1 interacts with beta-F1-ATPase mRNA and inhibits its translation. *J Cell Sci* (2010) 123(Pt 16):2685–96. doi:10.1242/jcs.065920
54. Willers IM, Martínez-Reyes I, Martínez-Diez M, Cuezva J. miR-127-5p targets the 3'UTR of human β-F1-ATPase mRNA and inhibits its translation. *Biochim Biophys Acta Bioenergetics* (2012) 1817(5):838–48. doi:10.1016/j.bbabio.2012.03.005
55. Sanchez-Cenizo L, Formentini L, Aldea M, Ortega AD, Garcia-Huerta P, Sanchez-Arago M, et al. Up-regulation of the ATPase inhibitory factor 1 (IF1) of the mitochondrial H+-ATP synthase in human tumors mediates the metabolic shift of cancer cells to a Warburg phenotype. *J Biol Chem* (2010) 285(33):25308–13. doi:10.1074/jbc.M110.146480
56. Garcia-Bermudez J, Cuezva JM. The ATPase inhibitory factor 1 (IF1): a master regulator of energy metabolism and of cell survival. *Biochim Biophys Acta* (2016) 1857:1167–82. doi:10.1016/j.bbabio.2016.02.004
57. Gledhill JR, Montgomery MG, Leslie AG, Walker JE. How the regulatory protein, IF(1), inhibits F(1)-ATPase from bovine mitochondria. *Proc Natl Acad Sci U S A* (2007) 104(40):15671–6. doi:10.1073/pnas.0707326104
58. Campanella M, Casswell E, Chong S, Farah Z, Wieckowski MR, Abramov AY, et al. Regulation of mitochondrial structure and function by the F1Fo-ATPase inhibitor protein, IF1. *Cell Metab* (2008) 8(1):13–25. doi:10.1016/j.cmet.2008.06.001
59. Garcia-Bermudez J, Sanchez-Arago M, Soldevilla B, Del Arco A, Nuevo-Tapioles C, Cuezva JM. PKA phosphorylates the ATPase inhibitory factor 1 and inactivates its capacity to bind and inhibit the mitochondrial H-ATP synthase. *Cell Rep* (2015) 12:2143–55. doi:10.1016/j.celrep.2015.08.052
60. Sanchez-Arago M, Formentini L, Martinez-Reyes I, Garcia-Bermudez J, Santacatterina F, Sanchez-Cenizo L, et al. Expression, regulation and clinical relevance of the ATPase inhibitory factor 1 in human cancers. *Oncogenesis* (2013) 2:e46. doi:10.1038/oncsis.2013.9
61. Formentini L, Sánchez-Aragó M, Sánchez-Cenizo L, Cuezva JM. The mitochondrial ATPase inhibitory factor 1 (IF1) triggers a ROS-mediated retrograde pro-survival and proliferative response. *Mol Cell* (2012) 45:731–42. doi:10.1016/j.molcel.2012.01.008
62. Esparza-Molto PB, Nuevo-Tapioles C, Cuezva JM. Regulation of the H+-ATP synthase by IF1: a role in mitohormesis. *Cell Mol Life Sci* (2017) 74(12):2151–66. doi:10.1007/s00018-017-2462-8
63. Gao YX, Chen L, Hu XG, Wu HB, Cui YH, Zhang X, et al. ATPase inhibitory factor 1 expression is an independent prognostic factor in non-small cell lung cancer. *Am J Cancer Res* (2016) 6(5):1141–8.
64. Wei S, Fukuhara H, Kawada C, Kurabayashi A, Furihata M, Ogura S, et al. Silencing of ATPase inhibitory factor 1 inhibits cell growth via cell cycle arrest in bladder cancer. *Pathobiology* (2015) 82(5):224–32. doi:10.1159/000439027
65. Wu J, Shan Q, Li P, Wu Y, Xie J, Wang X. ATPase inhibitory factor 1 is a potential prognostic marker for the migration and invasion of glioma. *Oncol Lett* (2015) 10(4):2075–80. doi:10.3892/ol.2015.3548
66. Zhang C, Min L, Liu J, Tian W, Han Y, Qu L, et al. Integrated analysis identified an intestinal-like and a diffuse-like gene sets that predict gastric cancer outcome. *Tumour Biol* (2016) 37(12):16317–35. doi:10.1007/s13277-016-5454-7
67. Garcia-Ledo L, Nuevo-Tapioles C, Cuevas-Martin C, Martinez-Reyes I, Soldevilla B, Gonzalez-Llorente L, et al. Overexpression of the ATPase inhibitory factor 1 favors a non-metastatic phenotype in breast cancer. *Front Oncol* (2017) 7:69. doi:10.3389/fonc.2017.00069
68. Kurbasic E, Sjostrom M, Krogh M, Folkesson E, Grabau D, Hansson K, et al. Changes in glycoprotein expression between primary breast tumour and synchronous lymph node metastases or asynchronous distant metastases. *Clin Proteomics* (2015) 12(1):13. doi:10.1186/s12014-015-9084-7
69. Song R, Song H, Liang Y, Yin D, Zhang H, Zheng T, et al. Reciprocal activation between ATPase inhibitory factor 1 and NF-kappaB drives hepatocellular carcinoma angiogenesis and metastasis. *Hepatology* (2014) 60:1659–73. doi:10.1002/hep.27312
70. Yin T, Lu L, Xiong Z, Wei S, Cui D. ATPase inhibitory factor 1 is a prognostic marker and contributes to proliferation and invasion of human gastric cancer cells. *Biomed Pharmacother* (2015) 70:90–6. doi:10.1016/j.biopha.2014.12.036
71. Santacatterina F, Sanchez-Cenizo L, Formentini L, Mobasher MA, Casas E, Rueda CB, et al. Down-regulation of oxidative phosphorylation in the liver by expression of the ATPase inhibitory factor 1 induces a tumor-promoter metabolic state. *Oncotarget* (2016) 7(1):490–508. doi:10.18632/oncotarget.6357
72. Formentini L, Pereira MP, Sanchez-Cenizo L, Santacatterina F, Lucas JJ, Navarro C, et al. In vivo inhibition of the mitochondrial H+-ATP synthase in neurons promotes metabolic preconditioning. *EMBO J* (2014) 33(7):762–78. doi:10.1002/embj.201386392
73. Formentini L, Santacatterina F, Nunez De Arenas C, Stamatakis K, Lopez-Martinez D, Logan A, et al. Mitochondrial ROS production protects the intestine from inflammation through functional M2 macrophage polarization. *Cell Rep* (2017) 19(6):1202–13. doi:10.1016/j.celrep.2017.04.036
74. Lehninger A. *Biochemistry*. New York: Worth Publishers (1970).
75. Sanchez-Arago M, Chamorro M, Cuezva JM. Selection of cancer cells with repressed mitochondria triggers colon cancer progression. *Carcinogenesis* (2010) 31(4):567–76. doi:10.1093/carcin/bgq012
76. Nazarewicz RR, Dikalova A, Bikineyeva A, Ivanov S, Kirilyuk IA, Grigor'ev IA, et al. Does scavenging of mitochondrial superoxide attenuate cancer prosurvival signaling pathways? *Antioxid Redox Signal* (2013) 19(4):344–9. doi:10.1089/ars.2013.5185
77. Porporato PE, Payen VL, Perez-Escuredo J, De Saedeleer CJ, Danhier P, Copetti T, et al. A mitochondrial switch promotes tumor metastasis. *Cell Rep* (2014) 8(3):754–66. doi:10.1016/j.celrep.2014.06.043
78. Faccenda D, Nakamura J, Gorini G, Dhoot GK, Piacentini M, Yoshida M, et al. Control of mitochondrial remodeling by the ATPase inhibitory factor 1 unveils a pro-survival relay via OPA1. *Cell Rep* (2017) 18(8):1869–83. doi:10.1016/j.celrep.2017.01.070
79. Denicola GM, Karreth FA, Humpton TJ, Gopinathan A, Wei C, Frese K, et al. Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. *Nature* (2011) 475(7354):106–9. doi:10.1038/nature10189
80. Matic I, Cocco S, Ferraina C, Martin-Jimenez R, Florenzano F, Crosby J, et al. Neuroprotective coordination of cell mitophagy by the F1Fo-ATPase inhibitory factor 1 (IF). *Pharmacol Res* (2015) 103:56–68. doi:10.1016/j.phrs.2015.10.010
81. Chin RM, Fu X, Pai MY, Vergnes L, Hwang H, Deng G, et al. The metabolite alpha-ketoglutarate extends lifespan by inhibiting ATP synthase and TOR. *Nature* (2014) 510(7505):397–401. doi:10.1038/nature13264
82. Fu X, Chin RM, Vergnes L, Hwang H, Deng G, Xing Y, et al. 2-hydroxyglutarate inhibits ATP synthase and mTOR signaling. *Cell Metab* (2015) 22(3):508–15. doi:10.1016/j.cmet.2015.06.009
83. Yan H, Parsons DW, Jin G, Mclendon R, Rasheed BA, Yuan W, et al. IDH1 and IDH2 mutations in gliomas. *N Engl J Med* (2009) 360(8):765–73. doi:10.1056/NEJMoa0808710
84. Gross S, Cairns RA, Minden MD, Driggers EM, Bittinger MA, Jang HG, et al. Cancer-associated metabolite 2-hydroxyglutarate accumulates in acute myelogenous leukemia with isocitrate dehydrogenase 1 and 2 mutations. *J Exp Med* (2010) 207(2):339–44. doi:10.1084/jem.20092506
85. Van Den Bent MJ, Dubbink HJ, Marie Y, Brandes AA, Taphoorn MJ, Wesseling P, et al. IDH1 and IDH2 mutations are prognostic but not predictive for outcome in anaplastic oligodendroglial tumors: a report of the European Organization for Research and Treatment of Cancer Brain Tumor Group. *Clin Cancer Res* (2010) 16(5):1597–604. doi:10.1158/1078-0432.CCR-09-2902

86. Hansen M, Hsu AL, Dillin A, Kenyon C. New genes tied to endocrine, metabolic, and dietary regulation of lifespan from a *Caenorhabditis elegans* genomic RNAi screen. *PLoS Genet* (2005) 1(1):119–28. doi:10.1371/journal.pgen.0010017
87. Sun X, Wheeler CT, Yolitz J, Laslo M, Alberico T, Sun Y, et al. A mitochondrial ATP synthase subunit interacts with TOR signaling to modulate protein homeostasis and lifespan in *Drosophila*. *Cell Rep* (2014) 8(6):1781–92. doi:10.1016/j.celrep.2014.08.022
88. Goldberg J, Currais A, Prior M, Fischer W, Chiruta C, Ratliff E, et al. The mitochondrial ATP synthase is a shared drug target for aging and dementia. *Aging Cell* (2018). doi:10.1111/acel.12715

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

MINI REVIEW
published: 15 May 2018
doi: 10.3389/fonc.2018.00160

Phosphoinositide 3-Kinase/Akt Signaling and Redox Metabolism in Cancer

Nikos Koundouros[1,2] *and George Poulogiannis*[1,2]*

[1] Department of Cancer Biology, Institute of Cancer Research, London, United Kingdom, [2] Division of Computational and Systems Medicine, Department of Surgery and Cancer, Imperial College London, London, United Kingdom

Metabolic rewiring and the consequent production of reactive oxygen species (ROS) are necessary to promote tumorigenesis. At the nexus of these cellular processes is the aberrant regulation of oncogenic signaling cascades such as the phosphoinositide 3-kinase and AKT (PI3K/Akt) pathway, which is one of the most frequently dysregulated pathways in cancer. In this review, we examine the regulation of ROS metabolism in the context of PI3K-driven tumors with particular emphasis on four main areas of research. (1) Stimulation of ROS production through direct modulation of mitochondrial bioenergetics, activation of NADPH oxidases (NOXs), and metabolic byproducts associated with hyperactive PI3K/Akt signaling. (2) The induction of pro-tumorigenic signaling cascades by ROS as a consequence of phosphatase and tensin homolog and receptor tyrosine phosphatase redox-dependent inactivation. (3) The mechanisms through which PI3K/Akt activation confers a selective advantage to cancer cells by maintaining redox homeostasis. (4) Opportunities for therapeutically exploiting redox metabolism in *PIK3CA* mutant tumors and the potential for implementing novel combinatorial therapies to suppress tumor growth and overcome drug resistance. Further research focusing on the multi-faceted interactions between PI3K/Akt signaling and ROS metabolism will undoubtedly contribute to novel insights into the extensive pro-oncogenic effects of this pathway, and the identification of exploitable vulnerabilities for the treatment of hyperactive PI3K/Akt tumors.

Keywords: reactive oxygen species, cancer, phosphoinositide 3-kinase/Akt signaling, oxidative stress, metabolism

OPEN ACCESS

Edited by:
Giuseppe Filomeni,
Danish Cancer Society, Denmark

Reviewed by:
Paolo Armando Gagliardi,
Universität Bern, Switzerland
Antonella Papa,
Monash University, Australia

***Correspondence:**
George Poulogiannis
george.poulogiannis@icr.ac.uk

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *11 March 2018*
Accepted: *26 April 2018*
Published: *15 May 2018*

Citation:
Koundouros N and Poulogiannis G (2018) Phosphoinositide 3-Kinase/Akt Signaling and Redox Metabolism in Cancer. Front. Oncol. 8:160. doi: 10.3389/fonc.2018.00160

INTRODUCTION

Tumorigenesis is a multi-step process involving the complex interplay of several biological processes that are highly dependent on the activation of pro-proliferative and pro-survival signaling cascades, accumulation of genetic aberrations, and adaptation to various microenvironmental stress conditions (1, 2). Underpinning these tumorigenic processes is the ability of cancer cells to alter their metabolism to promote nutrient synthesis or scavenging to support their high proliferation demands (3, 4). A major consequence of such extensive metabolic rewiring is the production of reactive oxygen species (ROS), which include both free radical molecules such as superoxide ($O_2^{\bullet-}$) and hydroxyl radicals ($^{\bullet}OH$), as well as non-free radical species, of which hydrogen peroxide (H_2O_2) is among the most prominent (5). Although elevated ROS levels can have detrimental consequences on cell viability through extensive damage of proteins, DNA, and organelles, the concomitant increase in antioxidant and detoxification capacities in cancer cells allows for redox

homeostasis, thus generating a tightly regulated system whereby ROS can promote tumorigenesis and cancer progression (6, 7). Specifically, there is renewed interest in examining the implications of redox balance on cancer cell proliferation and survival through the regulation of key signaling cascades such as the phosphoinositide 3-kinase and AKT (PI3K/Akt) pathway, which is of particular relevance as it controls many hallmarks of cancer (1, 8, 9). In this review, we will examine the emerging relationship between redox homeostasis and PI3K/Akt signaling, and discuss how their cross-regulation may promote cancer pathogenesis. In addition, we present and look with optimism opportunities toward a future therapeutic exploitation of redox homeostasis in tumors with enhanced PI3K/Akt activation.

THE PI3K/AKT PATHWAY AT A GLANCE

The intricacies of PI3K/Akt signaling have been extensively reviewed previously (10–12), and will only be briefly introduced here. Activation of receptor tyrosine kinases (RTKs) or G-protein-coupled receptors facilitate the recruitment of class I PI3Ks which phosphorylate phosphatidylinositol-(4,5) bisphosphate (PIP_2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). Oncogenic mutations in *PIK3CA*—the gene encoding the class I PI3K catalytic subunit p110α—are found in approximately one-third of human cancers and 40% of breast cancers (13, 14). The accumulation of PIP_3 allows for the localization of AKT to the plasma membrane and its subsequent activation following phosphorylation on serine 473 and threonine 308 by mTORC2 and PDK1, respectively. Negative regulation of the PI3K/Akt pathway is largely mediated by the phosphatase and tensin homolog (PTEN) through dephosphorylation of PIP_3 to PIP_2. AKT is an important downstream effector of oncogenic PI3K signaling and regulates several pathways, including inhibition of apoptosis, stimulation of mTORC1-dependent cell growth, and modulation of cellular metabolism (11). Glucose and glutamine are considered as essential nutrients for cancer cell proliferation, and PI3K/Akt signaling has been shown to regulate the cellular metabolism of both nutrients. Specifically, AKT may directly increase glucose uptake through activation of the glucose transport receptor GLUT1, and stimulate glycolysis by phosphorylating hexokinase 2 (11, 15). Moreover, activating mutations in *PIK3CA* render colorectal cancer cells more dependent on glutamine anaplerosis to replenish TCA cycle intermediates through upregulation of glutamate pyruvate transaminase 2, and glutamine deprivation significantly reduces the proliferation of *PIK3CA* mutant, but not wild type, cancer cells (16). Later work has also demonstrated the importance of Akt-independent signaling cascades associated with PI3K activation, with a particular focus on serum and glucocorticoid-regulated kinases (SGKs) (17).

REGULATION OF ROS PRODUCTION BY PI3K/AKT SIGNALING

Aberrant PI3K/Akt signaling drives many of the molecular mechanisms contributing to increased ROS levels through direct modulation of mitochondrial bioenergetics and activation of NADPH oxidases (NOXs), or indirectly through the production of ROS as a metabolic by-product (**Figure 1**) (6, 18). Mitochondria are a major source of cellular ROS, and these are largely derived from electron leakage at complexes I and III of the electron transport chain (6, 19). Complex I in particular has been shown to generate ROS through two mechanisms: the first involves reduction of O_2 by flavin mononucleotide following binding of NADH induced by high NADH/NAD^+ ratios, and the second is through reverse electron transport whereby excessive NADH is produced following reduction of NAD^+ from ubiquinol (6, 20). Interestingly, AKT has been shown to translocate to the mitochondrial matrix and inner membrane in a PI3K-dependent manner following IGF-1 stimulation (21). AKT may directly phosphorylate mitochondrial GSK-3β, reducing its activity and thus alleviating the negative regulation imposed on pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes, which have been reported to generate superoxide and H_2O_2 (19, 22, 23).

The activation of NOXs by PI3K/Akt signaling also contributes to higher ROS levels in cancer cells (**Figure 1**) (18). There are seven members of the NOX enzyme family (NOX1-5, DUOX1, and DUOX2) that are expressed across several tissue types including the liver, lung, and gastrointestinal tract (24). NOXs are composed of different subunits including $p67^{phox}$, $p47^{phox}$, $p40^{phox}$, $p22^{phox}$, RAC1, RAC2, and NOXO1/NOXA1 which facilitate the localization of the enzyme complex to the cytoplasm or plasma membrane (24). The main function of NOX enzymes was first ascertained in phagocytes as contributing to the respiratory burst through production of superoxide following electron transfer from NADPH to oxygen (25). The activity of these enzymes is also becoming increasingly associated with various hallmarks of cancer including angiogenesis and metastasis (26). The importance of PI3K/Akt signaling in activating NOXs has been demonstrated by specifically ablating NOX activity following treatment with the PI3K inhibitor wortmannin or knockout of *Akt1* (18, 27). In terms of cancer progression, PI3K-mediated activation of NOX is necessary to promote cell migration and chemotaxis in response to stimulants such as hepatocyte growth factor (HGF) and platelet-derived growth factor (PDGF) (28, 29). Migration of lung endothelial cells requires HGF-mediated activation of c-MET, which signals through the PI3K/Akt pathway and results in the accumulation of $p47^{phox}$/Rac1 in lamellipodia and localized production of H_2O_2 to the leading edge of the cell (29). Consequently, inhibition of the PI3K/Akt pathway also inhibits the translocation of NOX subunits and reduces ROS, hindering the metastatic potential of lung cancer cells, at least in part, by reducing expression of the metalloprotease MMP9 and the pro-metastatic miRNA miR-21 (29–31). Angiogenesis is necessary for tumor growth especially following metastasis, and NOX isoforms have been implicated in re-vascularization particularly in PI3K/Akt-hyperactive tumors (32). Vascular endothelial growth factor is the most potent stimulant of angiogenesis and can activate NOX isoforms either directly or indirectly through PI3K/Akt induction (32–34). The subsequent production of superoxide and H_2O_2 are necessary for the regulation of transcription factors, which promote angiogenesis, including NF-κB, MMPs, COX-2, and HIF-1α (32).

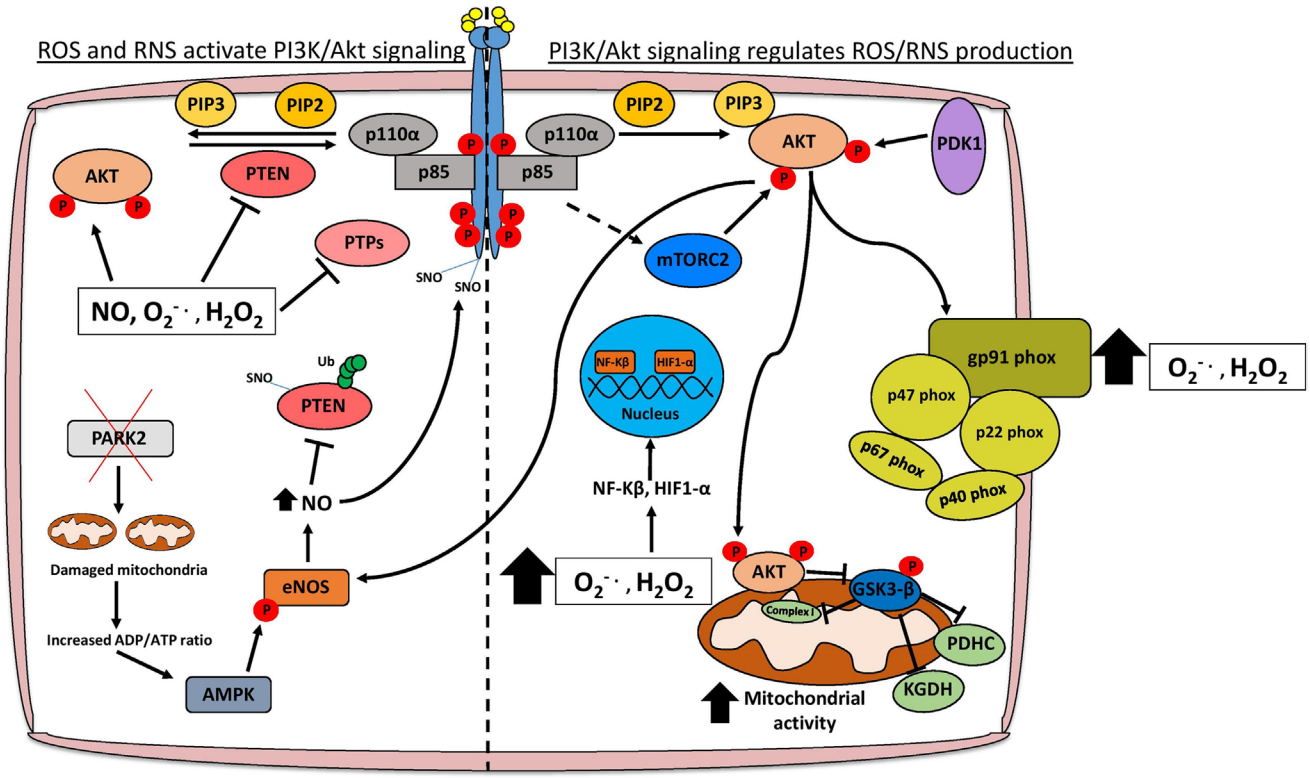


FIGURE 1 | Schematic representation of the interplay between PI3K/Akt signaling and redox stress. Activation of PI3K/Akt signaling occurs following stimulation of receptor tyrosine kinases (RTKs) or G-protein-coupled receptors. AKT is an important oncogenic effector of PI3K signaling and positively regulates reactive oxygen/nitrogen species (ROS/RNS) production through direct modulation of mitochondrial bioenergetics and activation of NADPH oxidases. Cellular ROS levels can also potentiate the PI3K cascade by directly activating AKT, stimulating RTKs through SNO, or through inhibition of various tumor suppressor proteins such as PTEN and PTPs. Moreover, elevated ROS levels promote the nuclear translocation of NF-κβ and HIF1-α which transcriptionally regulate several genes involved in cancer cell growth, proliferation, and survival. Abbreviations: PTP, protein tyrosine phosphatase; SNO, S-nitrosylation; $O_2^{\cdot-}$, superoxide; H_2O_2, hydrogen peroxide; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; Ub, ubiquitin; PDHC, pyruvate dehydrogenase complex; KGDH, alpha-ketoglutarate dehydrogenase; PTEN, phosphatase and tensin homolog; PI3K, phosphoinositide 3-kinase.

It is well established that hyperactive PI3K/Akt signaling contributes to increased glycolytic rate, and recent studies have identified dependencies on specific metabolic enzymes that, when perturbed, impact glycolytic flux and consequently cancer cell viability (35). Notably, *PIK3CA* mutant cancer cell lines have elevated 2-oxoglutarate dehydrogenase (OGDH) activity, and inhibition of this enzyme leads to reduced tumor growth *in vivo* (35). The sensitivity to OGDH inhibition can be explained by the observed decrease in the NAD^+/NADH ratio following accumulation of 2-oxoglutarate, thus limiting the available NAD^+ needed for glycolysis (35). Interestingly, OGDH is a potent source of ROS and this, together with the need to maintain a stable NAD^+/NADH ratio, suggests that maintaining redox homeostasis is essential for the proliferation of *PIK3CA* mutant tumors (35, 36). Another important ROS-generating metabolic pathway that is regulated by PI3K/Akt signaling is the production of prostaglandins (PGE_2) from arachidonic acid by the cyclooxegenases COX-1 and COX-2 (37). The peroxidase activity of the COX enzymes results in the generation of superoxide as a by-product (38). Recent clinical studies have also highlighted the importance of PGE_2 metabolism in PI3K-driven tumors, with *PIK3CA* mutation status being used as a biomarker for aspirin treatment in colorectal cancer (39). Thus, in addition to the direct effects of PI3K/Akt pathway in augmenting ROS levels, several indirect mechanisms associated with the intrinsic metabolic dependencies of *PIK3CA* mutant tumors exist, which could also contribute to the cellular pool of ROS levels.

ACTIVATION OF PI3K/AKT AND PRO-TUMORIGENIC SIGNALING BY ROS

Elevated pro-proliferative signaling cascades and inhibition of growth suppressors are necessary for tumorigenesis, and high ROS levels affect both, by potentiating activation of PI3K/Akt signaling mainly through inhibition of phosphatases such as PTEN or direct activation of oncogenes including AKT (40, 41). In breast cancer, exposure to hormones including 17-β estradiol and its derivative 4-OH-E2 lead to a dose-dependent increase in ROS levels and consequent malignant transformation of MCF10A cells *in vitro* and *in vivo* (42). Mechanistically, this is dependent on ROS-mediated hyper-phosphorylation of PI3K

and subsequent downstream PDK1-mediated activation of AKT, culminating in the upregulation of cell cycle promoting genes such as *CDK1* and *PCNA* (42). Interestingly, treatment with ROS scavengers such N-acetylcysteine (NAC) or knockdown of *AKT1* rescues the malignant transformation induced by 4-OH-E2 exposure, indicating an important regulatory role of ROS on PI3K and AKT (42). This regulation has also been demonstrated in T-cell acute lymphoblastic leukemia (T-ALL) where interleukin-7 (IL-7) enhances NOX and mitochondrial complex I activity, thus contributing to elevated ROS in T-ALL cells (43). AKT phosphorylation and activation is induced by the IL-7-dependent increase in ROS levels and promotes glucose uptake through upregulation of the GLUT1 receptor (43).

Excessive oxidative stress also activates PI3K/Akt signaling by inhibiting the activity of its negative regulator PTEN, one of the most frequently altered tumor suppressor genes in cancer (44). High ROS levels modulate PTEN through direct oxidation leading to reduced phosphatase activity, or indirectly through phosphorylation, which increases its stability and prevents its recruitment to the membrane (45, 46). For example, H_2O_2 mediated oxidation of cysteine residues 124 and 71 on PTEN leads to the reversible formation of disulfide bridges and reduction of its catalytic activity (47, 48). PTEN oxidation and subsequent inactivation can also occur in the absence of H_2O_2 treatment. This has been demonstrated in the COX-2- and LOX-5-dependent synthesis of prostaglandins from arachidonic acid in pancreatic cancer cells (49). Oxidized PTEN as a result of arachidonic acid metabolism does not migrate at a lower molecular weight under non-reducing conditions, whereas a migratory shift is seen following the formation of a disulfide bond between Cys-124 and Cys-71 upon H_2O_2 treatment (49). As COX-2 expression and activity is regulated by AKT, a model could exist in pancreatic tumors whereby arachidonic acid metabolism perpetuates PI3K/Akt activity through PTEN inactivation, consequently resulting in persistent prostaglandin production and associated inflammation (50, 51).

Oxidative stress can also induce posttranslational modifications, which facilitate PTEN ubiquitylation and subsequent degradation. One such redox-dependent modification, which contributes to PTEN inactivation and enhanced ubiquitin-proteasome degradation is S-nitrosylation (SNO) (52, 53). Mitochondrial dysfunction, which is triggered by loss of the tumor suppressor *PARK2* leads to a reduction in ATP levels and concomitant activation of AMPK (52, 54). AMPK-mediated activation of nitric oxide synthase 3 (NOS3/eNOS) leads to enhanced nitric oxide (NO) levels and NO-derived reactive nitrogen species, contributing to PTEN SNO on Cys-83 (52, 53, 55). This modification is important for the proliferation of PTEN proficient cells under energy-deprived conditions (52). Of note, the AKT kinase can also signal directly to activate eNOS, contributing to high NO levels (56). Nitrosative stress-induced posttranslational modifications, unlike in the case of PTEN, are not always limited to inhibitory effects, and can result in enhanced stabilization and activation of oncogenes including EGFR and Src that also contribute to the activation of the PI3K/Akt pathway (57). Taken together, these findings suggest a model whereby multiple oncogenic signaling cascades, in particular PI3K/Akt signaling, can be potentiated through elevated nitrosative stress to promote pro-survival adaptations during nutrient deprivation.

Reactive oxygen species can also modulate PI3K/Akt signaling by regulating protein tyrosine phosphatases (PTPs), which inhibit RTKs such as EGFR and PDGFR through dephosphorylation. PTPs can be broadly classified into four main groups based on their specific substrates and all contain an essential cysteine residue in the catalytic domain (58). ROS—and H_2O_2 in particular—have been shown to reversibly oxidize this cysteine residue, leading to reduced phosphatase activity of PTPs and sustained RTK activation (59). Notably, detoxification of H_2O_2 by overexpressing catalase or inhibition of NOXs contributes to a marked reduction in tyrosine phosphorylation of PDGFR, EGFR, and the insulin receptor (60, 61). Perhaps the most potent activator of PI3K/Akt signaling is insulin, and stimulation of IRS-1 following insulin binding, increases H_2O_2 generation and NOX4 activity (62). PTP1B and SHP-2 are the main PTPs that dephosphorylate IRS-1; therefore, their inhibition in response to insulin-induced ROS levels facilitates persistent activation of many downstream signaling cascades including the PI3K/Akt pathway (62). The implications of this regulation in the cancer context are not only limited to increased proliferation and metastatic potential, but also play a major role in promoting drug resistance (63, 64). The development of drug resistance is particularly relevant in breast cancer, where dysregulated IRS-1/IGF-1R signaling decreases the sensitivity to tamoxifen and trastuzumab in estrogen receptor (ER)- and HER2-positive tumors, respectively, by potentiating ERK and PI3K/Akt signaling (63, 65, 66). Thus, by inhibiting several key negative regulators, redox stress has a significant role in the activation of PI3K/Akt signaling and associated pro-tumorigenic phenotypes.

PI3K/AKT SIGNALING MAINTAINS REDOX BALANCE IN CANCER CELLS

In order for ROS to confer a selective advantage to cancer cells, there must be a concomitant increase in antioxidant responses to prevent adverse effects on cell viability. These responses include upregulation of the Keap1–Nrf2 pathway and modulation of glutathione metabolism, both of which are regulated by PI3K/Akt signaling (**Figure 2**). The transcription factor Nrf2 is recognized as a central mediator of ROS detoxification by inducing the expression of several enzymes that are involved in the antioxidant response, including glutathione *S*-transferase, superoxide dismutases, and NAD(P)H:quinone oxidoreductase 1 (Nqo1) (67). Under low concentrations of cellular ROS, Nrf2 is bound to the E3 ubiquitin ligase Keap1 in the cytosol and degraded by the proteasome (68). This interaction is inhibited under high concentrations of ROS, which oxidize cysteine residues on Keap1, thus allowing Nrf2 to translocate to the nucleus and induce the expression of genes containing antioxidant response elements (69). PI3K/Akt activation has been shown to be essential for the nuclear translocation of Nrf2, and accordingly treatment of neuroblastoma SH-SY5Y cells with PI3K inhibitors LY294002 and wortmannin, but not MAPK inhibitors, reduces

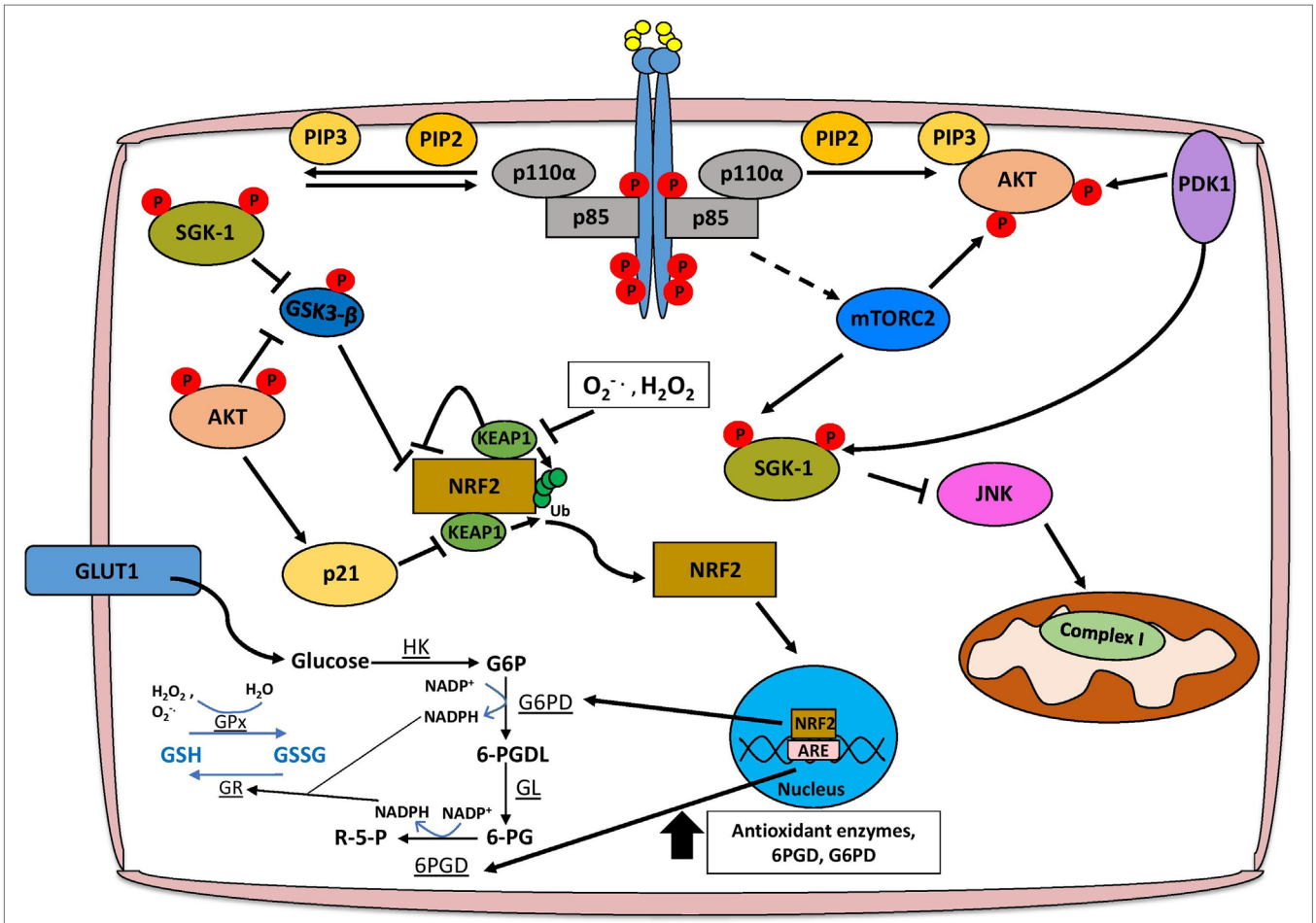


FIGURE 2 | PI3K/Akt signaling facilitates ROS detoxification and redox homeostasis. The concomitant increase in the cellular antioxidant response is necessary for redox homeostasis, and this is largely mediated through NRF2 signaling, glutathione metabolism, and production of NADPH. AKT facilitates the activation of NRF2 by inhibiting the interaction with KEAP1 and alleviating the negative regulation imposed by GSK3-β. Functional NRF2 translocates to the nucleus and transcriptionally activates genes involved in the antioxidant response such as glutathione S-transferase and superoxide dismutase, as well as the pentose phosphate pathway (PPP), which produces NADPH. Glutathione biosynthesis is an important antioxidant, which is regulated in a PI3K/Akt/Nrf2-dependent mechanism, and the conversion of glutathione between reduced (GSH) and oxidized (GSSG) forms is dependent on PPP-derived NADPH. Finally, Akt-independent signaling axes through SGK-1 may also promote ROS detoxification. Abbreviations: HK, hexokinase; G6P, glucose-6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; 6-PGDL, 6-phosphogluconolactone; GL, gluconolactonase; 6-PG, 6-phosphogluconate; 6-PGD, 6-phosphogluconate dehydrogenase; R-5-P, ribulose-5-phosphate; GR, glutathione reductase; GPx, glutathione peroxidase; SGK, serum and glucocorticoid-regulated kinase; PI3K, phosphoinositide 3-kinase.

Nrf2 transcriptional activation of antioxidant genes (70). One of the most important implications of PI3K-mediated upregulation of Nrf2 signaling is promoting cancer cell survival by conferring protection against excessive oxidative stress (71, 72). This is particularly relevant in BRCA1-deficient breast cancers, which lack effective DNA repair mechanisms through homologous recombination and are therefore susceptible to genetic modification induced by ROS (73). Interestingly, although up to 80% of BRCA1 mutant breast cancers are ER negative, estrogen positively regulates Nrf2 transcriptional activity through activation of PI3K/Akt signaling allowing BRCA1-null cells to detoxify high ROS levels and accumulate additional genetic aberrations that may contribute to tumorigenesis (73, 74). Inhibition of PI3K/Akt signaling either by PTEN overexpression or BKM120 treatment hinders estrogen-mediated Nrf2 activation, suggesting that targeting this pathway might be beneficial in treating BRCA1-deficient tumors by re-sensitizing them to elevated ROS levels (73).

Phosphoinositide 3-kinase/Akt activation has both Nrf2-dependent and -independent effects on cellular metabolism that contribute to ROS detoxification. Several antioxidant pathways rely on the reducing power of NADPH, which is predominantly generated by 6-phosphogluconate dehydrogenase (6PGD) and glucose-6-phosphate dehydrogenase (G6PD) from the pentose phosphate pathway (PPP) (75, 76). Active Nrf2 induces the expression of the aforementioned PPP enzymes through an AKT-dependent mechanism, as well as enzymes directly involved in NADPH synthesis such as malic enzyme 1 (ME1) and isocitrate dehydrogenase IDH (77). As AKT is a potent activator of glucose uptake and glycolysis, tumors dependent on PI3K/Akt signaling could shunt the glucose-6-phosphate (G6P) generated during

glycolysis to the PPP activated by Nrf2, thus maintaining a stable pool of NADPH, which could be used for anabolic processes to sustain tumor growth and proliferation, or ROS detoxification (77, 78) (**Figure 2**).

Phosphoinositide 3-kinase/Akt and Nrf2 signaling, as well as the associated increase in NADPH are key regulators of the synthesis of glutathione, which exists in either reduced (GSH) or oxidized (GSSG) form (79). Under conditions of oxidative stress, enzymes such as GSH transferase (GSH-Tr) and GSH peroxidase (GPx) facilitate the reduction of ROS, including H_2O_2, by oxidizing GSH to GSSG (79). In order to completely detoxify ROS, oxidized GSSG must then be reduced back to GSH by NADPH-dependent glutathione reductase (GSSG-Rx) (79). The induction of glutathione synthesis through upregulation of glutamate-L-cysteine ligase, as well as transcriptional activation of GSH-Tr and GPx is dependent on a PI3K/Akt/Nrf2 signaling axis (80, 81). AKT can also increase the stability of Nrf2 by activating p21Cip1/WAF1 which disrupts the interaction between Keap1 and Nrf2, and by inhibiting GSK-3β that leads to the reduction of Nrf2 phosphorylation, preventing its nuclear export and ubiquitination (82, 83). Importantly, glutathione biosynthesis has been shown to be a metabolic vulnerability in *PIK3CA* mutant breast cancer cells, as treatment of *PIK3CA* or *AKT* mutant MCF10A cells with buthionine sulfoximine (BSO) significantly reduces anchorage-independent growth and inhibits cell proliferation in 3D, but not 2D culture (83). Furthermore, cisplatin treatment when given in combination with BSO leads to tumor regression of *PIK3CA* mutant, but not wild type, cell line-derived xenograft models indicating that disrupting redox homeostasis through GSH metabolism could repurpose existing therapies for the treatment of *PIK3CA* mutant breast cancers (83).

Although AKT is perhaps the most extensively characterized downstream effector of PI3K signaling, recent studies have highlighted the importance of an Akt-independent axis which relies on other members of the AGC serine/threonine kinase family such as PDK1, RSK, and SGK1 (84, 85). In particular, SGK1 has been shown to promote antioxidant responses during pregnancy that are essential for fetal development, and exert protective effects in Parkinson's disease (86). SGK1 can negatively regulate JNK signaling, an important inducer of superoxide species by modulating complex I activity of the electron transport chain (87, 88). In addition, SGK1-mediated inactivation of GSK3-β facilitates MCL1 localization to the mitochondria to significantly reduce mitochondrial ROS production through inhibition of NOX4 expression, adversely affecting the response to chemotherapy (88, 89). It is important to note, however, that in models of lung cancer, MCL1 may also promote ROS production from the mitochondria by binding to voltage-dependent anion channels and increasing the mitochondrial flux of calcium (90).

THERAPEUTICALLY EXPLOITING REDOX HOMEOSTASIS IN PI3K-DEPENDENT TUMORS

In terms of redox homeostasis, it is clear that PI3K/Akt signaling is unique in that it activates both ROS generating and detoxifying processes, thus creating a stable presence of ROS, which can exert pro-tumorigenic effects. This raises the attractive therapeutic prospect of perturbing redox homeostasis in tumors with hyperactive PI3K/Akt signaling either alone or in combination with existing treatments. Chemotherapy and radiotherapy are among the most common therapeutic interventions for cancer patients, and one mechanism through which they induce apoptosis in cancer cells is by upregulating ROS (91). Anthracyclins, such as doxorubicin, and platinum-based therapies including cisplatin, can directly induce ROS production through modulation of the electron transport chain, and elevated ROS levels subsequently activate caspases, cytochrome *c* release, and DNA damage leading to apoptosis (92, 93). Previous clinical studies have demonstrated that *PIK3CA* mutant breast cancers display decreased sensitivity to anthracyclines and cisplatin, however, as these drugs exert anti-cancer effects through ROS upregulation, it is conceivable that the antioxidant pathways, which are elevated by PI3K/Akt signaling, could counteract these therapies (91, 94). Indeed, active Nrf2 protects cancer cells from ROS induced cell death, and inhibition of this transcription factor by brusatol treatment enhances the response to cisplatin (95, 96). In addition, targeting glutathione biosynthesis with BSO selectively sensitizes *PIK3CA* mutant breast tumors to cisplatin as compared to wild-type ones, indicating that impairing the antioxidant response could be an exploitable vulnerability in PI3K-driven tumors (83).

The apparent anti-cancer effects of excessive ROS accumulation are also important in targeting tumors, which have become resistant to PI3K/Akt inhibitors. A common strategy to overcome resistance is to place patients on "drug holidays" and subsequently re-challenge them with the inhibitor (97). In PI3K inhibitor resistant breast cancer cells, substantial metabolic rewiring occurs following removal of the class IA PI3K inhibitor GDC-0941 that is characterized by increased glycolysis and mitochondrial respiration (98). During drug holidays, the metabolic phenotype of resistant cancer cells is altered by an Akt-independent PI3K/mTORC1 signaling axis, which drives excessive production of ROS and inhibits the proliferation of these cells (98). Notably, this proliferative defect is rescued following treatment of resistant cells with ROS scavengers such as NAC, thus demonstrating the importance of regulating ROS homeostasis in prohibiting the expansion of a resistant cell population emerging through therapies (98). These findings present an opportunity to improve responses by specifically exploiting the unique metabolic profile of PI3K inhibitor resistant cells, either through glucose deprivation or by further increasing oxidative stress (98). Developing resistance to AKT-specific inhibitors such as MK2206 is also a significant clinical problem, and studies into the identification of synthetic lethal interactions between AKT and antioxidant inhibitors have shown promising results in overcoming drug resistance (99). In non-small cell lung carcinoma (NSCLC) models, dual treatment with the thioredoxin reductase-1 (TXNRD1) antioxidant inhibitor auranofin and MK2206 induced cancer cell-specific apoptosis through ROS-stimulated JNK signaling (99). Importantly, this synthetic lethality was observed in lung tumors with functional NRF2–KEAP1 signaling and overexpression of TXNRD1, indicating that the activity of the

anti-oxidative response in NSCLC could be used as a biomarker for determining which patients may benefit from dual AKT/TXNRD1 inhibition (99).

Although it seems that enhancing ROS production may have inhibitory effects on cancer cells, it is important to note that finding the balance between antioxidant and ROS-generating mechanisms is complex and so is their therapeutic exploitation (100). Notably, there is still significant debate regarding the administration of antioxidants during cancer therapy as a means of limiting drug toxicity and whether their use adversely affects the patient's response, and/or the potential systemic consequences of deliberately elevating ROS levels (101). It is, therefore, necessary to consider all of these implications and to ensure that tumors are well characterized at the genetic and metabolic levels to determine if targeting redox homeostasis is a suitable treatment option.

CONCLUSION AND FUTURE PERSPECTIVES

Several pro-tumorigenic processes converge on hyperactive PI3K/Akt signaling, and it is becoming increasingly evident that ROS metabolism is no exception to this. The capacity for the PI3K/Akt cascade to directly activate both ROS generating and various antioxidant pathways suggests a tight regulation on cellular redox homeostasis, the intricacies of which merit further investigation. Understanding the complex interplay between ROS and PI3K/Akt signaling is particularly relevant for developing therapeutic strategies to target tumors dependent on this pathway, especially since recent clinical trials have demonstrated only modest responses to PI3K/Akt pathway inhibitors and development of resistance (102). While ROS metabolism certainly adds another layer of complexity to PI3K/Akt signaling, this area of research holds great promise, not only for the potential identification of novel biomarkers and metabolic dependencies but also the prospect of implementing more potent therapeutic combinations, which perturb redox homeostasis and effectively target PI3K-driven tumors.

AUTHOR CONTRIBUTIONS

NK and GP wrote the manuscript and designed the figures.

FUNDING

NK is supported by an Institute of Cancer Research PhD studentship. Work in the GP lab is supported by the Institute of Cancer Research and a Cancer Research UK Grand Challenge award (C59824/A25044).

REFERENCES

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) 144(5):646–74. doi:10.1016/j.cell.2011.02.013
2. Tabassum DP, Polyak K. Tumorigenesis: it takes a village. *Nat Rev Cancer* (2015) 15(8):473–83. doi:10.1038/nrc3971
3. DeBerardinis RJ, Chandel NS. Fundamentals of cancer metabolism. *Sci Adv* (2016) 2(5):e1600200. doi:10.1126/sciadv.1600200
4. Pavlova NN, Thompson CB. The emerging hallmarks of cancer metabolism. *Cell Metab* (2016) 23(1):27–47. doi:10.1016/j.cmet.2015.12.006
5. Liou GY, Storz P. Reactive oxygen species in cancer. *Free Radic Res* (2010) 44(5):479–96. doi:10.3109/10715761003667554
6. Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J* (2009) 417:1–13. doi:10.1042/BJ20081386
7. Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. *Curr Biol* (2014) 24(10):R453–62. doi:10.1016/j.cub.2014.03.034
8. Fruman DA, Rommel C. PI3K and cancer: lessons, challenges and opportunities. *Nat Rev Drug Discov* (2014) 13(2):140–56. doi:10.1038/nrd4204
9. Irani K, Xia Y, Zweier JL, Sollott SJ, Der CJ, Fearon ER, et al. Mitogenic signaling mediated by oxidants in Ras-transformed fibroblasts. *Science* (1997) 275(5306):1649–52. doi:10.1126/science.275.5306.1649
10. Cantley LC. The phosphoinositide 3-kinase pathway. *Science* (2002) 296(5573):1655–7. doi:10.1126/science.296.5573.1655
11. Fruman DA, Chiu H, Hopkins BD, Bagrodia S, Cantley LC, Abraham RT. The PI3K pathway in human disease. *Cell* (2017) 170(4):605–35. doi:10.1016/j.cell.2017.07.029
12. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer* (2002) 2(7):489–501. doi:10.1038/nrc839
13. Campbell IG, Russell SE, Choong DY, Montgomery KG, Ciavarella ML, Hooi CS, et al. Mutation of the PIK3CA gene in ovarian and breast cancer. *Cancer Res* (2004) 64(21):7678–81. doi:10.1158/0008-5472.CAN-04-2933
14. Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature* (2012) 490(7418):61–70. doi:10.1038/nature11412
15. Elstrom RL, Bauer DE, Buzzai M, Karnauskas R, Harris MH, Plas DR, et al. Akt stimulates aerobic glycolysis in cancer cells. *Cancer Res* (2004) 64(11):3892–9. doi:10.1158/0008-5472.CAN-03-2904
16. Hao Y, Samuels Y, Li Q, Krokowski D, Guan BJ, Wang C, et al. Oncogenic PIK3CA mutations reprogram glutamine metabolism in colorectal cancer. *Nat Commun* (2016) 7:11971. doi:10.1038/ncomms11971
17. Vasudevan KM, Barbie DA, Davies MA, Rabinovsky R, McNear CJ, Kim JJ, et al. AKT-independent signaling downstream of oncogenic PIK3CA mutations in human cancer. *Cancer Cell* (2009) 16(1):21–32. doi:10.1016/j.ccr.2009.04.012
18. Chatterjee S, Browning EA, Hong NK, DeBolt K, Sorokina EM, Liu WD, et al. Membrane depolarization is the trigger for PI3K/Akt activation and leads to the generation of ROS. *Am J Physiol Heart Circ Physiol* (2012) 302(1):H105–14. doi:10.1152/ajpheart.00298.2011
19. Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ. Production of reactive oxygen species by mitochondria: central role of complex III. *J Biol Chem* (2003) 278(38):36027–31. doi:10.1074/jbc.M304854200
20. Jastroch M, Divakaruni AS, Mookerjee S, Treberg JR, Brand MD. Mitochondrial proton and electron leaks. *Essays Biochem* (2010) 47:53–67. doi:10.1042/bse0470053
21. Bijur GN, Jope RS. Rapid accumulation of Akt in mitochondria following phosphatidylinositol 3-kinase activation. *J Neurochem* (2003) 87(6):1427–35. doi:10.1046/j.1471-4159.2003.02113.x
22. Li C, Li Y, He L, Agarwal AR, Zeng N, Cadenas E, et al. PI3K/AKT signaling regulates bioenergetics in immortalized hepatocytes. *Free Radic Biol Med* (2013) 60:29–40. doi:10.1016/j.freeradbiomed.2013.01.013
23. Starkov AA, Fiskum G, Chinopoulos C, Lorenzo BJ, Browne SE, Patel MS, et al. Mitochondrial alpha-ketoglutarate dehydrogenase complex generates reactive oxygen species. *J Neurosci* (2004) 24(36):7779–88. doi:10.1523/JNEUROSCI.1899-04.2004
24. Panday A, Sahoo MK, Osorio D, Batra S. NADPH oxidases: an overview from structure to innate immunity-associated pathologies. *Cell Mol Immunol* (2015) 12(1):5–23. doi:10.1038/cmi.2014.89
25. Forman HJ, Torres M. Reactive oxygen species and cell signaling: respiratory burst in macrophage signaling. *Am J Respir Crit Care Med* (2002) 166(12 Pt 2):S4–8. doi:10.1164/rccm.2206007
26. Meitzler JL, Antony S, Wu Y, Juhasz A, Liu H, Jiang G, et al. NADPH oxidases: a perspective on reactive oxygen species production in tumor biology. *Antioxid Redox Signal* (2014) 20(17):2873–89. doi:10.1089/ars.2013.5603

27. Chen Q, Powell DW, Rane MJ, Singh S, Butt W, Klein JB, et al. Akt phosphorylates p47phox and mediates respiratory burst activity in human neutrophils. *J Immunol* (2003) 170(10):5302–8. doi:10.4049/jimmunol.170.10.5302
28. Baumer AT, Ten Freyhaus H, Sauer H, Wartenberg M, Kappert K, Schnabel P, et al. Phosphatidylinositol 3-kinase-dependent membrane recruitment of Rac-1 and p47phox is critical for alpha-platelet-derived growth factor receptor-induced production of reactive oxygen species. *J Biol Chem* (2008) 283(12):7864–76. doi:10.1074/jbc.M704997200
29. Usatyuk PV, Fu PF, Mohan V, Epshtein Y, Jacobson JR, Gomez-Cambronero J, et al. Role of c-Met/phosphatidylinositol 3-kinase (PI3k)/Akt signaling in hepatocyte growth factor (HGF)-mediated lamellipodia formation, reactive oxygen species (ROS) generation, and motility of lung endothelial cells. *J Biol Chem* (2014) 289(19):13476–91. doi:10.1074/jbc.M113.527556
30. Chen JS, Wang Q, Fu XH, Huang XH, Chen XL, Cao LQ, et al. Involvement of PI3K/PTEN/AKT/mTOR pathway in invasion and metastasis in hepatocellular carcinoma: association with MMP-9. *Hepatol Res* (2009) 39(2):177–86. doi:10.1111/j.1872-034X.2008.00449.x
31. Yan S, Liu G, Pei C, Chen W, Li P, Wang Q, et al. Inhibition of NADPH oxidase protects against metastasis of human lung cancer by decreasing microRNA-21. *Anticancer Drugs* (2015) 26(4):388–98. doi:10.1097/CAD.0000000000000198
32. Ushio-Fukai M, Frey RS, Fukai T, Malik AB. Reactive oxygen species and endothelial permeability. *Free Radic Eff Membr* (2008) 61:147–89. doi:10.1016/S1063-5823(08)00208-1
33. Abid R, Guo SD, Minami T, Spokes KC, Ueki K, Skurk C, et al. Vascular endothelial growth factor activates PI3K/Akt/forkhead signaling in endothelial cells. *Arterioscl Thromb Vasc Biol* (2004) 24(2):294–300. doi:10.1161/01.ATV.0000110502.10593.06
34. Ruan GX, Kazlauskas A. VEGF-A engages at least three tyrosine kinases to activate PI3K/Akt. *Cell Cycle* (2012) 11(11):2047–8. doi:10.4161/cc.20535
35. Ilic N, Birsoy K, Aguirre AJ, Kory N, Pacold ME, Singh S, et al. PIK3CA mutant tumors depend on oxoglutarate dehydrogenase. *Proc Natl Acad Sci U S A* (2017) 114(17):E3434–43. doi:10.1073/pnas.1617922114
36. Mailloux RJ, Gardiner D, O'Brien M. 2-Oxoglutarate dehydrogenase is a more significant source of O-2(·-)/H_2O_2 than pyruvate dehydrogenase in cardiac and liver tissue. *Free Radic Biol Med* (2016) 97:501–12. doi:10.1016/j.freeradbiomed.2016.06.014
37. Bhattacharyya A, Chattopadhyay R, Mitra S, Crowe SE. Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiol Rev* (2014) 94(2):329–54. doi:10.1152/physrev.00040.2012
38. Kukreja RC, Kontos HA, Hess ML, Ellis EF. Pgh synthase and lipoxygenase generate superoxide in the presence of Nadh or Nadph. *Circ Res* (1986) 59(6):612–9. doi:10.1161/01.RES.59.6.612
39. Liao XY, Lochhead P, Nishihara R, Morikawa T, Kuchiba A, Yamauchi M, et al. Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. *N Engl J Med* (2012) 367(17):1596–606. doi:10.1056/NEJMoa1207756
40. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. *Nat Rev Cancer* (2011) 11(2):85–95. doi:10.1038/nrc2981
41. Niwa K, Inanami O, Yamamori T, Ohta T, Hamasu T, Kuwabara M. Redox regulation of PI3K/Akt and p53 in bovine aortic endothelial cells exposed to hydrogen peroxide. *Antioxid Redox Signal* (2003) 5(6):713–22. doi:10.1089/152308603770380016
42. Okoh VO, Felty Q, Parkash J, Poppiti R, Roy D. Reactive oxygen species via redox signaling to PI3K/AKT pathway contribute to the malignant growth of 4-hydroxy estradiol-transformed mammary epithelial cells. *PLoS One* (2013) 8(2):e54206. doi:10.1371/journal.pone.0054206
43. Silva A, Girio A, Cebola I, Santos CI, Antunes F, Barata JT. Intracellular reactive oxygen species are essential for PI3K/Akt/mTOR-dependent IL-7-mediated viability of T-cell acute lymphoblastic leukemia cells. *Leukemia* (2011) 25(6):960–7. doi:10.1038/leu.2011.56
44. Yuan TL, Cantley LC. PI3K pathway alterations in cancer: variations on a theme. *Oncogene* (2008) 27(41):5497–510. doi:10.1038/onc.2008.245
45. Kang KH, Lemke G, Kim JW. The PI3K-PTEN tug-of-war, oxidative stress and retinal degeneration. *Trends Mol Med* (2009) 15(5):191–8. doi:10.1016/j.molmed.2009.03.005
46. Vazquez F, Grossman SR, Takahashi Y, Rokas MV, Nakamura N, Sellers WR. Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex. *J Biol Chem* (2001) 276(52):48627–30. doi:10.1074/jbc.C100556200
47. Kwon J, Lee SR, Yang KS, Ahn Y, Kim YJ, Stadtman ER, et al. Reversible oxidation and inactivation of the tumor suppressor PTEN in cells stimulated with peptide growth factors. *Proc Natl Acad Sci U S A* (2004) 101(47):16419–24. doi:10.1073/pnas.0407396101
48. Lee SR, Yang KS, Kwon J, Lee C, Jeong W, Rhee SG. Reversible inactivation of the tumor suppressor PTEN by H_2O_2. *J Biol Chem* (2002) 277(23):20336–42. doi:10.1074/jbc.M111899200
49. Covey TM, Edes K, Fitzpatrick FA. Akt activation by arachidonic acid metabolism occurs via oxidation and inactivation of PTEN tumor suppressor. *Oncogene* (2007) 26(39):5784–92. doi:10.1038/sj.onc.1210391
50. Glynn SA, Prueitt RL, Ridnour LA, Boersma BJ, Dorsey TM, Wink DA, et al. COX-2 activation is associated with Akt phosphorylation and poor survival in ER-negative, HER2-positive breast cancer. *BMC Cancer* (2010) 10:626. doi:10.1186/1471-2407-10-626
51. St-Germain ME, Gagnon V, Mathieu I, Parent S, Asselin E. Akt regulates COX-2 mRNA and protein expression in mutated-PTEN human endometrial cancer cells. *Int J Oncol* (2004) 24(5):1311–24. doi:10.3892/ijo.24.5.1311
52. Gupta A, Anjomani-Virmouni S, Koundouros N, Dimitriadi M, Choo-Wing R, Valle A, et al. PARK2 depletion connects energy and oxidative stress to PI3K/Akt activation via PTEN S-nitrosylation. *Mol Cell* (2017) 65(6):999–1013.e7. doi:10.1016/j.molcel.2017.02.019
53. Kwak YD, Ma T, Diao SY, Zhang X, Chen YM, Hsu J, et al. NO signaling and S-nitrosylation regulate PTEN inhibition in neurodegeneration. *Mol Neurodegener* (2010) 5:49. doi:10.1186/1750-1326-5-49
54. Mihaylova MM, Shaw RJ. The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. *Nat Cell Biol* (2011) 13(9):1016–23. doi:10.1038/ncb2329
55. Schulz E, Schuhmacher S, Munzel T. When metabolism rules perfusion AMPK-mediated endothelial nitric oxide synthase activation. *Circ Res* (2009) 104(4):422–4. doi:10.1161/CIRCRESAHA.109.194274
56. Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, et al. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt (vol 399, pg 597, 1999). *Nature* (1999) 400(6746):792. doi:10.1038/23523
57. Switzer CH, Glynn SA, Cheng RYS, Ridnour LA, Green JE, Ambs S, et al. S-nitrosylation of EGFR and Src activates an oncogenic signaling network in human basal-like breast cancer. *Mol Cancer Res* (2012) 10(9):1203–15. doi:10.1158/1541-7786.MCR-12-0124
58. Alonso A, Sasin J, Bottini N, Friedberg I, Friedberg I, Osterman A, et al. Protein tyrosine phosphatases in the human genome. *Cell* (2004) 117(6):699–711. doi:10.1016/j.cell.2004.05.018
59. Meng TC, Fukada T, Tonks NK. Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo. *Mol Cell* (2002) 9(2):387–99. doi:10.1016/S1097-2765(02)00445-8
60. Bae YS, Kang SW, Seo MS, Baines IC, Tekle E, Chock PB, et al. Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. *J Biol Chem* (1997) 272(1):217–21. doi:10.1074/jbc.272.1.217
61. Sundaresan M, Yu ZX, Ferrans VJ, Irani K, Finkel T. Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. *Science* (1995) 270(5234):296–9. doi:10.1126/science.270.5234.296
62. Goldstein BJ, Mahadev K, Wu X, Zhu L, Motoshima H. Role of insulin-induced reactive oxygen species in the insulin signaling pathway. *Antioxid Redox Signal* (2005) 7(7–8):1021–31. doi:10.1089/ars.2005.7.1021
63. Denduluri SK, Idowu O, Wang Z, Liao Z, Yan Z, Mohammed MK, et al. Insulin-like growth factor (IGF) signaling in tumorigenesis and the development of cancer drug resistance. *Genes Dis* (2015) 2(1):13–25. doi:10.1016/j.gendis.2014.10.004
64. Pollak MN, Schernhammer ES, Hankinson SE. Insulin-like growth factors and neoplasia. *Nat Rev Cancer* (2004) 4(7):505–18. doi:10.1038/nrc1387
65. Gallardo A, Lerma E, Escuin D, Tibau A, Munoz J, Ojeda B, et al. Increased signalling of EGFR and IGF1R, and deregulation of PTEN/PI3K/Akt pathway are related with trastuzumab resistance in HER2 breast carcinomas. *Br J Cancer* (2012) 106(8):1367–73. doi:10.1038/bjc.2012.85
66. Massarweh S, Osborne CK, Creighton CJ, Qin L, Tsimelzon A, Huang S, et al. Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function. *Cancer Res* (2008) 68(3):826–33. doi:10.1158/0008-5472.CAN-07-2707

67. Kansanen E, Kuosmanen SM, Leinonen H, Levonen AL. The Keap1-Nrf2 pathway: mechanisms of activation and dysregulation in cancer. *Redox Biol* (2013) 1(1):45–9. doi:10.1016/j.redox.2012.10.001
68. Taguchi K, Yamamoto M. The KeAP1-NRF2 system in cancer. *Front Oncol* (2017) 7:85. doi:10.3389/fonc.2017.00085
69. Menegon S, Columbano A, Giordano S. The dual roles of NRF2 in cancer. *Trends Mol Med* (2016) 22(7):578–93. doi:10.1016/j.molmed.2016.05.002
70. Nakaso K, Yano H, Fukuhara Y, Takeshima T, Wada-Isoe K, Nakashima K. PI3K is a key molecule in the Nrf2-mediated regulation of antioxidative proteins by hemin in human neuroblastoma cells. *FEBS Lett* (2003) 546(2–3):181–4. doi:10.1016/S0014-5793(03)00517-9
71. De Nicola GM, Karreth FA, Humpton TJ, Gopinathan A, Wei C, Frese K, et al. Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. *Nature* (2011) 475(7354):106–9. doi:10.1038/nature10189
72. Sporn MB, Liby KT. NRF2 and cancer: the good, the bad and the importance of context. *Nat Rev Cancer* (2012) 12(8):564–71. doi:10.1038/nrc3278
73. Gorrini C, Gang BP, Bassi C, Wakeham A, Baniasadi SP, Hao Z, et al. Estrogen controls the survival of BRCA1-deficient cells via a PI3K-NRF2-regulated pathway. *Proc Natl Acad Sci U S A* (2014) 111(12):4472–7. doi:10.1073/pnas.1324136111
74. Foulkes WD, Metcalfe K, Sun P, Hanna WM, Lynch HT, Ghadirian P, et al. Estrogen receptor status in BRCA1- and BRCA2-related breast cancer: the influence of age, grade, and histological type. *Clin Cancer Res* (2004) 10(6):2029–34. doi:10.1158/1078-0432.CCR-03-1061
75. Fernandez-Marcos PJ, Nobrega-Pereira S. NADPH: new oxygen for the ROS theory of aging. *Oncotarget* (2016) 7(32):50814–5. doi:10.18632/oncotarget.10744
76. Patra KC, Hay N. The pentose phosphate pathway and cancer. *Trends Biochem Sci* (2014) 39(8):347–54. doi:10.1016/j.tibs.2014.06.005
77. Mitsuishi Y, Taguchi K, Kawatani Y, Shibata T, Nukiwa T, Aburatani H, et al. Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. *Cancer Cell* (2012) 22(1):66–79. doi:10.1016/j.ccr.2012.05.016
78. Jiang P, Du W, Wu M. Regulation of the pentose phosphate pathway in cancer. *Protein Cell* (2014) 5(8):592–602. doi:10.1007/s13238-014-0082-8
79. Lu SC. Regulation of glutathione synthesis. *Mol Aspects Med* (2009) 30(1–2):42–59. doi:10.1016/j.mam.2008.05.005
80. Chanas SA, Jiang Q, McMahon M, McWalter GK, McLellan LI, Elcombe CR, et al. Loss of the Nrf2 transcription factor causes a marked reduction in constitutive and inducible expression of the glutathione S-transferase Gsta1, Gsta2, Gstm1, Gstm2, Gstm3 and Gstm4 genes in the livers of male and female mice. *Biochem J* (2002) 365:405–16. doi:10.1042/bj20020320
81. Hayes JD, Chanas SA, Henderson CJ, McMahon M, Sun C, Moffat GJ, et al. The Nrf2 transcription factor contributes both to the basal expression of glutathione S-transferases in mouse liver and to their induction by the chemopreventive synthetic antioxidants, butylated hydroxyanisole and ethoxyquin. *Biochem Soc Trans* (2000) 28:33–41. doi:10.1042/bst0280033
82. Chen WM, Sun Z, Wang XJ, Jiang T, Huang ZP, Fang DY, et al. Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response. *Mol Cell* (2009) 34(6):663–73. doi:10.1016/j.molcel.2009.04.029
83. Lien EC, Lyssiotis CA, Juvekar A, Hu H, Asara JM, Cantley LC, et al. Glutathione biosynthesis is a metabolic vulnerability in PI(3)K/Akt-driven breast cancer. *Nat Cell Biol* (2016) 18(5):572–8. doi:10.1038/ncb3341
84. Arencibia JM, Pastor-Flores D, Bauer AF, Schulze JO, Biondi RM. AGC protein kinases: from structural mechanism of regulation to allosteric drug development for the treatment of human diseases. *Biochim Biophys Acta* (2013) 1834(7):1302–21. doi:10.1016/j.bbapap.2013.03.010
85. Pearce LR, Komander D, Alessi DR. The nuts and bolts of AGC protein kinases. *Nat Rev Mol Cell Biol* (2010) 11(1):9–22. doi:10.1038/nrm2822
86. Salker MS, Christian M, Steel JH, Nautiyal J, Lavery S, Trew G, et al. Deregulation of the serum- and glucocorticoid-inducible kinase SGK1 in the endometrium causes reproductive failure. *Nat Med* (2011) 17(11):1509–13. doi:10.1038/nm.2498
87. Chambers JW, LoGrasso PV. Mitochondrial c-Jun N-terminal kinase (JNK) signaling initiates physiological changes resulting in amplification of reactive oxygen species generation. *J Biol Chem* (2011) 286(18):16052–62. doi:10.1074/jbc.M111.223602
88. Iqbal S, Howard S, LoGrasso PV. Serum- and glucocorticoid-inducible kinase 1 confers protection in cell-based and in in vivo neurotoxin models via the c-Jun N-terminal kinase signaling pathway. *Mol Cell Biol* (2015) 35(11):1992–2006. doi:10.1128/MCB.01510-14
89. Demelash A, Pfannenstiel LW, Liu L, Gastman BR. Mcl-1 regulates reactive oxygen species via NOX4 during chemotherapy-induced senescence. *Oncotarget* (2017) 8(17):28154–68. doi:10.18632/oncotarget.15962
90. Huang H, Shah K, Bradbury NA, Li C, White C. Mcl-1 promotes lung cancer cell migration by directly interacting with VDAC to increase mitochondrial Ca2+ uptake and reactive oxygen species generation. *Cell Death Dis* (2014) 5:e1482. doi:10.1038/cddis.2014.419
91. Manda G, Isvoranu G, Comanescu MV, Manea A, Butuner BD, Korkmaz KS. The redox biology network in cancer pathophysiology and therapeutics. *Redox Biol* (2015) 5:347–57. doi:10.1016/j.redox.2015.06.014
92. Conklin KA. Chemotherapy-associated oxidative stress: impact on chemotherapeutic effectiveness. *Integr Cancer Ther* (2004) 3(4):294–300. doi:10.1177/1534735404270335
93. Johnson TM, Yu ZX, Ferrans VJ, Lowenstein RA, Finkel T. Reactive oxygen species are downstream mediators of p53-dependent apoptosis. *Proc Natl Acad Sci U S A* (1996) 93(21):11848–52. doi:10.1073/pnas.93.21.11848
94. Liedtke C, Cardone L, Tordai A, Yan K, Gomez HL, Figureoa LJ, et al. PIK3CA-activating mutations and chemotherapy sensitivity in stage II-III breast cancer. *Breast Cancer Res* (2008) 10(2):R27. doi:10.1186/bcr1984
95. Ren D, Villeneuve NF, Jiang T, Wu T, Lau A, Toppin HA, et al. Brusatol enhances the efficacy of chemotherapy by inhibiting the Nrf2-mediated defense mechanism. *Proc Natl Acad Sci U S A* (2011) 108(4):1433–8. doi:10.1073/pnas.1014275108
96. Singh A, Bodas M, Wakabayashi N, Bunz F, Biswal S. Gain of Nrf2 function in non-small-cell lung cancer cells confers radioresistance. *Antioxid Redox Signal* (2010) 13(11):1627–37. doi:10.1089/ars.2010.3219
97. Kuczynski EA, Sargent DJ, Grothey A, Kerbel RS. Drug rechallenge and treatment beyond progression – implications for drug resistance. *Nat Rev Clin Oncol* (2013) 10(10):571–87. doi:10.1038/nrclinonc.2013.158
98. Dermit M, Casado P, Rajeeve V, Wilkes EH, Foxler DE, Campbell H, et al. Oxidative stress downstream of mTORC1 but not AKT causes a proliferative defect in cancer cells resistant to PI3K inhibition. *Oncogene* (2017) 36(19):2762–74. doi:10.1038/onc.2016.435
99. Dai B, Yoo SY, Bartholomeusz G, Graham RA, Majidi M, Yan S, et al. KEAP1-dependent synthetic lethality induced by AKT and TXNRD1 inhibitors in lung cancer. *Cancer Res* (2013) 73(17):5532–43. doi:10.1158/0008-5472.CAN-13-0712
100. Chandel NS, Tuveson DA. The promise and perils of antioxidants for cancer patients. *N Engl J Med* (2014) 371(2):177–8. doi:10.1056/NEJMcibr1405701
101. Ladas EJ, Jacobson JS, Kennedy DD, Teel K, Fleischauer A, Kelly KM. Antioxidants and cancer therapy: a systematic review. *J Clin Oncol* (2004) 22(3):517–28. doi:10.1200/JCO.2004.03.086
102. Massacesi C, Di Tomaso E, Urban P, Germa C, Quadt C, Trandafir L, et al. PI3K inhibitors as new cancer therapeutics: implications for clinical trial design. *Onco Targets Ther* (2016) 9:203–10. doi:10.2147/OTT.S89967

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 14 August 2018
doi: 10.3389/fonc.2018.00272

Computational Structural Biology of *S*-nitrosylation of Cancer Targets

Emmanuelle Bignon[1], *Maria Francesca Allega*[1], *Marta Lucchetta*[1], *Matteo Tiberti*[1] and *Elena Papaleo*[1,2]*

[1] Computational Biology Laboratory, Danish Cancer Society Research Center, Copenhagen, Denmark, [2] Translational Disease Systems Biology, Faculty of Health and Medical Sciences, Novo Nordisk Foundation Center for Protein Research, University of Copenhagen, Copenhagen, Denmark

Nitric oxide (NO) plays an essential role in redox signaling in normal and pathological cellular conditions. In particular, it is well known to react *in vivo* with cysteines by the so-called *S*-nitrosylation reaction. *S*-nitrosylation is a selective and reversible post-translational modification that exerts a myriad of different effects, such as the modulation of protein conformation, activity, stability, and biological interaction networks. We have appreciated, over the last years, the role of *S*-nitrosylation in normal and disease conditions. In this context, structural and computational studies can help to dissect the complex and multifaceted role of this redox post-translational modification. In this review article, we summarized the current state-of-the-art on the mechanism of *S*-nitrosylation, along with the structural and computational studies that have helped to unveil its effects and biological roles. We also discussed the need to move new steps forward especially in the direction of employing computational structural biology to address the molecular and atomistic details of *S*-nitrosylation. Indeed, this redox modification has been so far an underappreciated redox post-translational modification by the computational biochemistry community. In our review, we primarily focus on *S*-nitrosylated proteins that are attractive cancer targets due to the emerging relevance of this redox modification in a cancer setting.

Keywords: *S*-nitrosylation, (de)nitrosylating enzymes, redox modifications, molecular dynamics simulations, cysteine, redox cancer biology

OPEN ACCESS

Edited by:
Michael Breitenbach,
University of Salzburg, Austria

Reviewed by:
Andrey V. Kozlov,
Ludwig Boltzmann Gesellschaft, Austria
Mario Schubert,
University of Salzburg, Austria

***Correspondence:**
Elena Papaleo
elenap@cancer.dk

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *18 April 2018*
Accepted: *02 July 2018*
Published: *14 August 2018*

Citation:
Bignon E, Allega MF, Lucchetta M, Tiberti M and Papaleo E (2018) Computational Structural Biology of S-nitrosylation of Cancer Targets. Front. Oncol. 8:272. doi: 10.3389/fonc.2018.00272

INTRODUCTION

Despite being amino acids (Cys) play diverse roles in biology. In fact, they represent a special class of residues due to the thiol moiety of their side chain (**Figure 1**). The thiol group can undergo a plethora of different biological modifications affecting protein structure, reactivity, stability and function (1). Cysteines are thus unique molecular switches (2). These modifications include, for example, disulfide bridge formation, high oxidation states, sulfenylation, persulfidation, metalation, *S*-nitrosylation, glutathionylation, sulfhydratation, among others. Cysteines can also be impacted by lipid modifications? including palmitoylation and prenylation or they can be the coordinating residues for metal ions such as zinc, iron, or copper in metallo-proteins.

Oxidative modifications of cysteine thiols can be a reversible or irreversible processes. Examples of reversible Cys modifications include Cys sulfenylation, *S*-glutathionylation and *S*-nitrosylation (SNO). With regards to the latter, NO is a reactive gas produced by NO synthases (NOS) 1–3 using the substrate arginine. NO produced inside the cell can be efficiently and quickly consumed through reactions with (bio)molecules that are in proximity to the NO source (3). A significant amount of the NO signal can be stored and propagated as nitrosyl adducts at specific cysteine sites of proteins via *S*-nitrosylation.

In this review, we will focus on computational structural and chemical studies that helped the understanding of the complex mechanisms induced by *S*-nitrosylation, as well as possible future directions for the computational studies of *S*-nitrosylation, which is a rather underappreciated modification compared to other well-known and more investigated post-translational modifications (PTMs), such as phosphorylation.

At first, we will provide the general background of what *S*-nitrosylation is, what is known on the enzymes regulating this PTM, as well as which are the biological effects so far discovered to be triggered by *S*-nitrosylation. We will then discuss the recent approaches and findings from bioinformatics, computational chemistry, and biochemistry methods applied to *S*-nitrosylation with particular attention to targets of interest for cancer research.

S-NITROSYLATION OF CYSTEINE RESIDUES

Among the broad spectrum of cysteine redox modifications mentioned above, *S*-nitrosylation (SNO) accounts for the oxidative modification of cysteines by nitric oxide (NO) to form *S*-nitrosothiols (4). SNO is, by far, the reversible Cys modification with the most significant prevalence and cellular functions, providing a ubiquitous mechanism for cellular signaling mediated by thiols. NO exerts its primary biological functions through protein *S*-nitrosylation so that it can be considered the prototype of redox-based signals (4, 5).

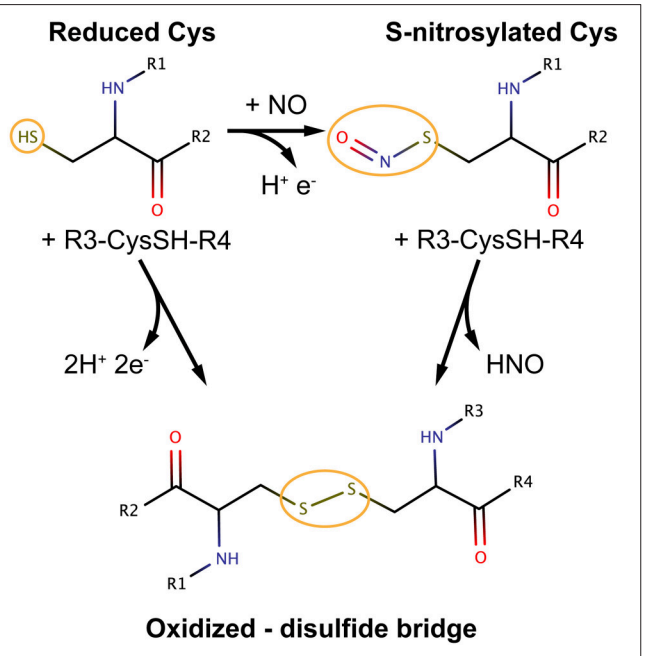


FIGURE 1 | Most relevant cysteine oxidation states in the context of *S*-nitrosylation and possible pathways of interconversion. The figure is meant to be illustrative and does not depict the complete reaction schemes. R groups are part of the protein main chain.

S-nitrosylation is considered a reversible and ubiquitous PTM and many studies demonstrated its role in protein activity, stability, localization, and protein-protein interactions (see section Biological Mechanisms Promoted by *S*-nitrosylation) in a myriad of cellular processes (6). *S*-nitrosylation emerged in the last decades as a new paradigm in signal transduction and regulation of proteins (7). More than 3,000 proteins are known to be affected by this redox PTM (3).

Alterations of *S*-nitrosylated protein targets or the enzymes regulating SNO have been also associated with different pathologies, including cancer, cardiovascular, respiratory, and neurodegenerative disorders (4, 6, 8, 9).

Protein *S*-nitrosylation features tight spatiotemporal specificity for certain protein Cys residue (10, 11). If physiological amounts of NO are present, only one or few Cys residues of a protein are targeted. These modifications are generally sufficient to change the protein function, activity or specificity for interaction partners (11–13). *S*-nitrosylation, as well as other alternative *S*-oxidative modifications mediated by reactive oxygen species, can target separated populations of Cys residues. Thus, depending on the Cys that is modified, the functional effects that are triggered can be very diverse (14). *S*-nitrosylation has also been shown to target distinct Cys residues with the final goal of exerting a coordinated effect (4). The known and possible determinants of specificity of *S*-nitrosylation toward certain Cys residues are discussed more in details in section Computational Structural and Chemical Studies of *S*-nitrosylation. Computational studies allowed to disclose them and define sequence or structural motifs around SNO sites.

One major mechanism promoted by *S*-nitrosylation of proteins is based on *S*-transnitrosylation, i.e., the capability of modifying with SNO other protein targets thus allowing the propagation of the SNO-based signals (4), similarly to the well-known kinase-based signaling cascade.

Different degrees of *S*-nitrosylation can be observed in protein targets, spanning from cases of mono-SNO (single cysteine) to multiple cysteines (multi-SNO), depending on the availability of NO, as well as on the properties of the target proteins in the proximity of the Cys sites (3). NO production is tightly controlled in normal conditions, and this leads to a basal *S*-nitrosylation level. In this context, a subset of SNO targets (such as CD40 and pro-caspases) is in their resting state. Another subset of proteins, on the contrary, is constitutively *S*-nitrosylated. Indeed, they require this modification to be active, as exemplified by caveolin-1 or connexin-43. In cases in which these targets have multiple SNO sites, an incremental degree of *S*-nitrosylation can be observed with transitions from mono- to poly-SNO which allow a progressive activation in response to stress (3). The degree of SNO and the switch from mono- to multi-SNO can also strongly depend on the availability and accessibility of cysteines

in the target protein. Cysteines are, for example, enriched in proteins that are on the cell surface or actively involved in cell-to-cell communication and signal transduction, such as CD40, other TNFRs, receptor tyrosine kinases, integrins, and connexins (3).

Due to the high SNO reactivity and the propensity for *S*-transnitrosylation, to fully appreciate the mechanisms and consequences of *S*-nitrosylation in normal and cancer cellular contexts is particularly challenging. Hence, it becomes crucial to assess the role of SNO-proteins as both targets of this redox modification and transducers of the SNO signal.

ENZYMATIC REGULATION OF *S*-NITROSYLATION

Cellular *S*-nitrosylation is dynamically governed by the equilibrium between *S*-nitrosylated proteins and low-molecular-weight *S*-nitrosothiols, which in turns are tightly controlled by several enzymes, such as S-nitrosylases and denitrosylases (4). NO is generally the product of three main isoforms of NO synthase (NOS) in mammalian cells (see section Nitric Oxide Synthases). *S*-nitrosothiols (RSNOs) are initially formed via different chemical routes that involve a one-electron oxidation, such as reaction of NO with thiyl radicals, transfer of the NO group from metal-NO complexes to a Cys thiolate, or the reaction of a Cys thiolate with species generated by NO auto-oxidation (4). Recent evidence suggested, however, an important role for metalloproteins in catalyzing *de novo S*-nitrosylation [see ref. (4) for more details]. The NO group is then transferred from a donor to an acceptor Cys thiol via *S*-transnitrosylation, (see section *S*-nitrosylation of Cysteine Residues). *S*-nitrosylation occurs not only in proteins but also in low molecular weight (LMW) thiols such as glutathione (GSH) and coenzyme A. SNO-proteins and SNO-LMW thiols exist in thermodynamic equilibria, which are regulated by the removal of the SNO moiety by the direct action of denitrosylases on the protein targets, as well as by the action of GSNOR on SNO-LMW molecules (see sections Denitrosylation Systems, Thioredoxin System and GSNOR System). These enzymes, described in the next sections, are crucial to control steady-state levels of SNO and to ensure the regulation of the NO-based signal cascade.

Nitric Oxide Synthases

The compartmentalization of SNO targets with NOS enzymes favors the interaction between the enzymes and the *S*-nitrosylation substrate which can, in turn, occur directly, or through scaffolding proteins (4). For example, the CAPON protein acts as a scaffold to mediate the interaction between nNOS and the *S*-nitrosylation target Dexras1 (15). Another example is the complex formed by iNOS and S100A8/A9, for which targeted *S*-transnitrosylation is favored by S100A9 on multiple protein targets that share a short linear motif I/L-X-C-X2-D/E where C represents the SNO site (4).

Each NOS isoform can also be *S*-nitrosylated, generally through an auto-catalytic mechanism, which involves a metal center. NOSs can then act as *S*-transnitrosylating partners for scaffolding proteins with or without the intervention of a LMW-SNO (4).

The different NOS isoforms are expressed in various organs, tissues, cells, or even subcellular compartments (16). For example, the localization of eNOS in the Golgi apparatus allows the generation of a local NO pool that specifically targets the compartmentalized proteins for *S*-nitrosylation (17). An example of another finely tuned compartmentalization is attested by the binding of different NOS variants to separate regions of the same target protein, which result in *S*-nitrosylation of different Cys residues (4).

Denitrosylation Systems

As mentioned in section S-nitrosylation of Cysteine Residues, *S*-nitrosylation is a PTM consisting of a covalent bond formation between of nitric oxide (NO) to a cysteine residue to form a *S*-nitrosothiol (5). In response to a specific stimulus, *S*-nitrosylated proteins (SNO-proteins) can undergo the reverse reaction of denitrosylation (i.e., reducing SNO back to SH). Two mechanisms of denitrosylation have been identified (18): the S-nitrosoglutathione reductase (GSNOR) system, comprising GSH and GSNOR, and the thioredoxin (Trx) system, comprising Trx and Trx reductase (TrxR) as shown in **Figure 2**. Both mechanisms use intermediate molecules (GSH and Trx) to remove the NO group from *S*-nitrosylated proteins. In the next paragraphs, we will illustrate these two denitrosylating systems and the cellular processes that they affect.

Thioredoxin System

Thioredoxins (Trx) are a family of small redox proteins involved in multiple cellular processes.

The Trx system was originally identified as a key player in the cellular redox homeostasis thanks to its role as a disulfide reductase (19). There are two distinct mammalian thioredoxins, Trx1- mainly localized in the cytosol but possibly translocated to the nucleus-and Trx2, mainly located in the mitochondria.

The thioredoxin system is composed of a thioredoxin (Trx), a thioredoxin reductase (TrxR), and NADPH. It includes the dithiol Cys-X-X-Cys active site that is essential for their oxidoreductase function (20). In the denitrosylation process, the reduced Trx [$Trx(SH)_2$] denitrosylates *S*-nitrosylated proteins forming a reduced protein thiol (-SH) and producing nitroxyl (HNO) or free NO groups and oxidized Trx [Trx(S-S)]; the latter is then reduced by TrxR at the expense of NADPH (21). For example, Trx1 reduces the target protein disulfide bond with concomitant oxidation of its Cys32 and Cys35 residues. TrxR then reduces the oxidized Trx1 thanks to NADPH consumption to regenerate the reduced Trx1 form.

Apart from its canonical role as a disulfide reductase, the Trx system has an important role in denitrosylation of *S*-nitrosylated proteins (21, 22). The Trx-dependent denitrosylation requires a multi-step process that entails: (i) the formation of mixed disulfide bridges through the attack of the nucleophilic Cys (Cys32 in Trx1) on the sulfur atom of the SNO moiety, (ii) the release of HNO, (iii) the resolution of the mixed disulfide bridge through the action of the second reactive Cys (Cys35 in Trx1) so

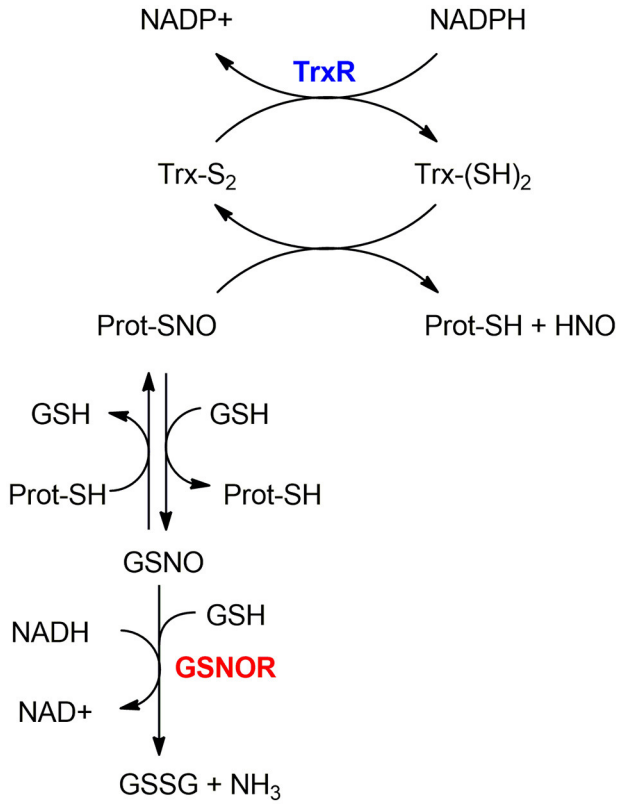


FIGURE 2 | Mechanisms of denitrosylation. Thioredoxin-system (top): The reduced Thioredoxin (Trx $(SH)_2$) denitrosylates the *S*-nitrosylated protein (Prot-SNO), releasing Prot-SH and the oxidized Trx (Trx(S_2)), which is further reduced back to (Trx $(SH)_2$) by Thioredoxin Reductase TrxR in a NADPH-dependent reaction. GSNOR-system (bottom): Prot-SNO is denitrosylated by *S*-transnitrosylation with glutathione (GSH), forming a reduced protein thiol (Prot-SH) and GSNO. The latter is then reduced by GSNOR to GSSG at the expenses of NADH and one GSH molecule. The resulting GSSG is further reduced by the Glutaredoxin enzyme (Grx) to give back the reduced GSH molecules—this step is not depicted here.

that the oxidized variant of Trx can be formed and (iv) the final reduction step by the action of TrxR (19). The knowledge of this mechanism was also exploited experimentally to entrap a protein target in its *S*-nitrosylated state mutating the resolving Cys of Trx (Cys35 in Trx1) (23, 24).

An alternative mechanism for Trx-mediated *S*-denitrosylation has also been proposed in which the initial step is postulated to be a *S*-transnitrosylation reaction from the target protein to the active site cysteine of Trx, with the subsequent release of HNO (24).

Another protein, i.e., the thioredoxin-interacting protein (TXNIP), regulates the Trx denitrosylating activity. In a feedback loop, NO controls this inhibition by repressing TXNIP activity, providing a dynamic regulation of Trx-mediated denitrosylation in response to NO levels (25).

Moreover, attesting the versatility of the Trx system, Trx1 has been identified as a *S*-transnitrosylase (26). Trx1, in the oxidized state, can be modified by *S*-nitrosylation on its Cys73, which in turn mediates the *S*-nitrosylation of caspase-3 and other protein targets (19). The SNO-Trx form can be then regulated by either the Trx system or a GSH-mediated denitrosylation (21). In agreement with the existence of an acid-base motif for *S*-nitrosylation (see section *S*-nitrosylation Sites Specificity), it has been shown that charged residues in the proximity of Cys73 are required for Trx transnitrosylase activity (27). The stability of SNO-Trx is regulated by both Trx- and GSH-mediated denitrosylation (21). Additional Trx1 *S*-transnitrosylation motifs have been proposed that involve proximal alanine residues (28).

A thioredoxin-related protein of 14 kDa called Trp14 was also recently identified to act as a denitrosylase for the other master regulator of *S*-nitrosylation, i.e., GSNOR, as well as certain SNO-proteins, including caspase-3 and cathepsin B (24). Trp14 activity tightly depends on both TrxR1 and NADPH even if further studies are needed to better clarify Trp14 role *in vivo*.

Studies on targets of Trx-mediated denitrosylation allowed to identify two motifs, C-X5-K and C-X6-K, within the SNO targets that are modulated by the Trx system (28). These motifs could be used to predict and identify new targets where the Trx system is the major regulator of the *S*-nitrosylated state.

GSNOR System

GSNOR (i.e., the GSNO reductase) is present in all mammals and ubiquitously expressed across different tissues (29). It is known as a class III alcohol dehydrogenase and it is encoded by the ADH5 human gene. GSNOR is a homodimer composed by two identical subunits (chains A and B) containing two bound zinc ions each (30).

The GSNOR-mediated protein denitrosylation requires the tripeptide GSH to form a reduced protein thiol (Prot-SH) and GSNO (18). GSNO is reduced by GSNOR to GSSG in a NADH-dependent reaction (using NADH as an electron donor) (31) involving hydride transfer. GSSG is further reduced by the Glutaredoxin enzyme (Grx) to give GSH back.

GSNOR expression and activity can be regulated by different biomolecules in a very context-dependent manner. For example, VEGF or IL-13 can induce GSNOR mRNA expression in lungs. Sp1 can transcriptionally regulate GSNOR levels in hepatocytes. MicroRNAs (miRs) such as miR-342-3p can downregulate GSNOR expression (4). Moreover, as also mentioned above, GSNOR is post-translationally regulated by *S*-nitrosylation and this modification has been suggested to regulate allosterically the enzyme activity, as attested by an enhanced GSNOR activity upon *S*-nitrosylation in mouse models (4).

The *S*-nitrosylated sites of GSNOR *in vivo* have not been identified yet and further studies will be required to fully understand and appreciate the mechanisms of this redox modification of the major regulator of *S*-nitrosylation. Prediction methods such as the ones described in section Prediction and Annotation of *S*-nitrosylated Proteins and the usage of molecular dynamics (MD) simulations could help in the identification of GSNO SNO-sites and in unveiling the determinants of its allosteric regulation. For example, the integration of computational techniques inspired by network theory and all-atom simulations hold promise to study long-range effects induced by PTMs (32–35). These methods could

thus be translated to the study of GSNOR *S*-nitrosylation as soon as accurate physical models for *S*-nitrosylation will be available and validated.

As mentioned before, GSNOR has been found in different kind of tissues, particularly in liver, brain and kidney. GSNOR deficiency might positively or negatively affect physiology. GSNOR is the only ADH enzyme in the brain, highlighting its importance in this organ. As a result of its ubiquitous expression in different tissues, GSNOR controls several molecular processes and is likely to be involved in disease conditions onset.

GSNOR in Cancer

Nitric oxide (NO) regulates protein functions, as well as the activity of many enzymes. *S*-nitrosylation is a key mechanism in the transmission of NO-based cellular signals in vital cellular processes such as DNA repair (36), apoptosis (37), cell proliferation, and cell cycle regulation. These are all processes related to cancer onset.

As a key enzyme of denitrosylation, GSNOR controls the intracellular levels of *S*-nitrosylated proteins and the reduction of its expression or stability has been shown to result in dysfunctional *S*-nitrosylation signaling and, eventually, in pathological states such as cancer (38). In particular, GSNOR deregulation has been observed to be involved in some pathways representing the hallmarks of cancer like DNA damage repair, energetic metabolism and cell death. Noteworthy, GSNOR tumor suppressor role has been recently proposed (38).

It was demonstrated that GSNOR-deficiency is sufficient to induce spontaneous formation of hepatocellular carcinoma (HCC) (39) through *S*-nitrosylation and proteasomal degradation of the key DNA repair protein O(6)-alkylguanine-DNA alkyltransferase (AGT). The AGT enzyme removes the alkyl group from guanine bases, repairing the highly mutagenic and cytotoxic O6-alkylguanines which can be generated by carcinogenic compounds such as the diethylnitrosamine (DEN). Alkylation affects the stability of AGT resulting in its irreversible inactivation through degradation via proteasome. A similar effect is induced by *S*-nitrosylation of AGT on its Cys145; *S*-nitrosylated AGT is still degraded via the proteasome, but in this case before the repair of O6-alkylguanines. By GSNO catabolism, GSNOR maintains low levels of *S*-nitrosylated AGT. Therefore, GSNOR deficiency inactivates AGT-dependent DNA repair and may critically contribute to hepatocarcinogenesis (38).

Besides DNA alteration, tumor cells reorganize their core metabolism to sustain their growth and proliferation and GSNOR involvement in metabolic pathways has been observed. In particular, GSNOR deficiency in hepatocytes is characterized by mitochondrial alteration and by increases in succinate dehydrogenase (SDH) levels (40). Succinate dehydrogenase - or respiratory complex II - participates in both the citric acid cycle and the electron transport chain and catalyzes the oxidation of succinate to fumarate, regulating the levels of these two metabolites that are included in the class of "oncometabolites." More precisely, the mechanism through which GSNOR ablation modulates SDH involves the mitochondrial molecular chaperone TNF receptor-associated protein 1 (TRAP1) which, if *S*-nitrosylated at Cys501, undergoes proteasomal degradation and is not able to interact with SDH, loosing the ability of inhibiting it (41). Besides, it was demonstrated that GSNOR ablation makes HCC cells more sensitive to SDH-targeting drugs (40), suggesting a new potential therapeutic target.

Different subtypes of breast cancers are also linked to the GSNOR expression modulation. In particular, the HER2 breast cancer subtype—characterized by high human epidermal growth factor receptor 2 (HER2) expression—is associated with lower expression of GSNOR which leads to a poor prognosis (42). Cañas et al. demonstrated that the antiproliferative effect of trastuzumab, a monoclonal antibody used for HER2 breast cancer, is suppressed by inhibition of GSNOR. Indeed, GSNOR restores the activation of survival signaling pathways, representing a possible reason for drug resistance observed in many patients on whom this treatment was used (43). Due to the central role of GSNOR in *S*-nitrosylation regulation and cancer, more insight will be gained in the future by comprehensive analyses of genomics and proteomics profiling of samples from cancer patients using biostatistics and bioinformatics to unveil the complex interplay between GSNOR, its regulators and targets in a cancer context.

BIOLOGICAL MECHANISMS PROMOTED BY *S*-NITROSYLATION

As mentioned above, *S*-nitrosylation exerts a plethora of functions inside the cell. Indeed, *S*-nitrosylation gained attention as a PTM, but it is still less understood at the molecular level compared to other well-known PTMs. In 1992, Stamler et al. proposed for the first time that the formation of biologically active *S*-nitrosothiols—more stable than NO itself—could represent an important mechanism through which NO is involved in the regulation of cellular activities (44).

In the context of tumor biology, we know that nitric oxide (NO) has different effects on cellular ability to survive and proliferate depending on its concentration. In fact, high concentrations of NO (>500 nM) appear to be toxic for cancer cells—causing cytostasis and apoptosis—whereas low concentrations (<100 nM) are able to induce the activation of cancer-promoting pathways (45). Thus, NO concentration—depending on the balance between NOSs activity and de-nitrosylation (see section GSNOR System)—determines also the extent of *S*-nitrosylation inside the cell. Another important source of NO is nitrite (46), especially in ischemic conditions but it is not the main focus of this section. In this section, we will try to summarize the polyhedral/multifaceted consequences of this PTM on protein function (**Figure 3**).

Influence of *S*-nitrosylation on Catalytic Activity of Enzymes

Probably one of the most common outcomes of S-nitrosylation is the regulation of catalytic activity of enzymes, whose modification can have activatory or inhibitory effects. In this way,

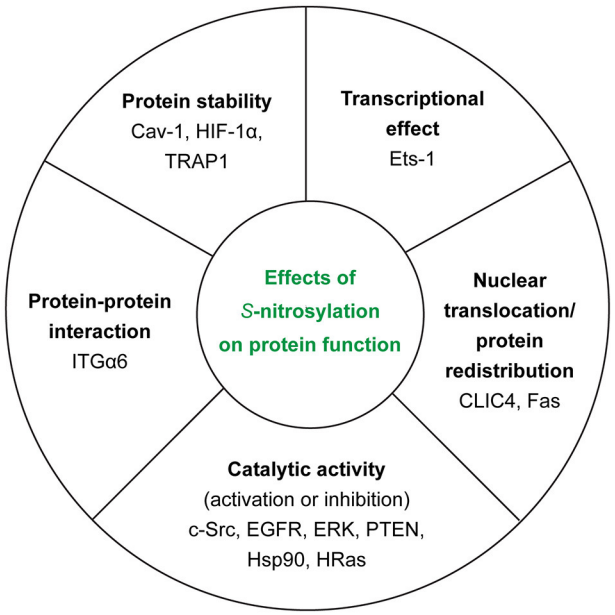


FIGURE 3 | Effects of *S*-nitrosylation on protein function. For each class, we reported the examples of the target proteins that we discussed in the main text.

the cellular redox state—highly variable/deregulated in cancer cells—exerts its role in controlling enzymatic activity.

S-nitrosylation can alter well-known cancer-related proteins, such as kinases. It has been observed in breast cancer cells that estrogens are able to work in synergy with NO to induce their proliferation and migration. In fact, in MCF7 cells, β-estradiol induces NOS expression and NO production, thus promoting the activation of c-Src through *S*-nitrosylation of Cys498 (47). c-Src is a tyrosine kinase, whose activation is responsible for disrupting E-cadherin junctions, promoting cell invasion and its catalytic activity is well-known to be regulated by phosphorylation. Recent evidence brought to light the existence of a tight regulation of c-Src based on *S*-nitrosylation and its cross-talk with phosphorylation (47).

The increase of iNOS (inducible NOS, also known as NOS2) is correlated with a decreased survival of ER negative and basal-like breast cancer (BC) patients. Indeed, this correlates with high EGFR phosphorylation levels. A study from 2012 showed that, in the context of basal-like BC, *S*-nitrosylation can stimulate EGFR and Src proteins (both membrane-associated proteins). The *S*-nitrosylated variants of EGFR and Src are then responsible for activating the oncogenic signaling based on c-Myc, Akt, STAT3, and β-catenin, while inhibiting the tumor suppressor PP2A (48). Moreover, NO levels required to activate these proteins are the same levels promoting an aggressive cellular phenotype (49).

More recently, it has been discovered that the NO-dependent EGFR activation can induce Extracellular signal-Regulated Kinase (ERK) phosphorylation—whose abnormal elevation has been described in tumor cells—thus activating it in basal-like triple negative breast cancer (50). ERK belongs to the MAPK superfamily and its aberrant upregulation and activation, which frequently occur in human tumors, are responsible for the acquisition of a malignant phenotype (51). In particular, there is a subset of basal-like BC, i.e., the BL2 molecular subtype, that is highly dependent on growth factor signaling (EGFR included) (52). In the BL2 subtype, Garrido et al. (50) demonstrated that NO-mediated activation of both EGFR and ERK was responsible for the increased migration and invasion abilities of cancer cells, this being accompanied by NF-kB activation and the increased secretion of pro-inflammatory cytokines.

ERK *S*-nitrosylation also links NO signaling to apoptosis. Indeed, ERK1 and ERK2 exert anti-apoptotic functions and are able, on the one hand, to induce the activity of other apoptosis antagonists, as for instance Bcl-2 and IAP. On the other hand, they can repress pro-apoptotic proteins, such as Bad and BIM (53). More in details, in a cellular model of NO-induced apoptosis, it has been demonstrated that NO decreases the levels of p-ERK, suggesting the *S*-nitrosylation of the kinase as an essential way to trigger cell death of tumor cells (54).

Recently, Gupta et al. (55) showed that *S*-nitrosylation plays a role also in the PI3K/Akt pathway, which is often dysregulated in cancer. In conditions of energy deprivation and in the presence of a signal able to activate the AMPK kinase, eNOS activation promotes inhibition of PTEN through its *S*-nitrosylation and degradation mediated by the ubiquitin-proteasome system. Intriguingly, PTEN inactivation upon *S*-nitrosylation was originally identified in the context of neurodegenerative diseases (56), whereas Gupta and coworkers, for the first time, demonstrated the role of this redox-dependent modification in supporting proliferation and survival of cancer cells through the activation of PI3K/Akt signaling and the subsequent stimulation of mTOR activity (55).

Heat Shock Protein 90 (Hsp90)—the cytosolic molecular chaperone—represents a co-activator of eNOS associating to the NO synthase together with Akt. Indeed, to exert this role, Hsp90 needs to work as an ATPase, but this activity was discovered to be inhibited by *S*-nitrosylation, occurring on Cys597, localized in the C-terminal domain in the region interacting with eNOS. In fact, it was proposed that the PTM induces a conformational change able to disrupt the interaction between eNOS and Hsp90. This may represent a mechanism to react to overproduction of NO inside the cells (57, 58). The *S*-nitrosylation of Cys597 also regulates ATP hydrolysis and chaperone activity of Hsp90 and shifts the conformational equilibrium within the ATPase cycle (58). Future studies in which the SNO modification of Cys597 can be properly modeled, with the related conformational changes assessed, will provide more details on the mechanism and interplay between this redox modification and the activation of the chaperone.

The class of Ras GTPases—HRas, NRas, and KRas, i.e., the founding members of a large superfamily of small GTPases—regulates several cytoplasmic signaling networks that govern cell growth, survival, and differentiation and act upstream to several pathways mentioned above (59). The three Ras proteins are over-activated by somatic mutations in 33% of human cancers, contributing to excessive growth, invasion, and ability to metastasize (60). The mutated variants of these proteins are

associated with a constitutively active GTP-bound state, which makes cancer cells addicted to the expression of oncogenic Ras proteins. Some years ago, it has been also demonstrated that HRas undergoes *S*-nitrosylation on Cys118 with the effect of stabilizing the GTP-bound HRas form by enhancing the dissociation of guanine nucleotides (61). For this reason, it has been proposed that this PTM could represent a way to diversify the Ras-dependent oncogenic signaling beyond that of the mutated Ras.

In summary, many examples have been provided of regulation of enzymatic activity by *S*-nitrosylation, and they are especially important in the context of kinases, G-coupled proteins and chaperones i.e., three usual suspects in a cancer setting.

Nuclear Translocation/Protein Redistribution

A large body of literature investigates and explains the multi-faceted role of ion channels, both potassium and chloride, in the process of tumorigenesis. Indeed, they are involved in many of the main processes leading the transformation of a normal cell into a neoplastic one and, among all the ion channels, several chloride intracellular channels (CLICs) have been demonstrated to be overexpressed in different cancer types (62). Among the latter, CLIC4 has been originally identified as a p53- and cMyc-responsive protein with proapoptotic functions, most of which are dependent on its translocation to the nucleus. Moreover, this channel undergoes structural conformational changes upon cellular redox changes (63, 64). In 2010, Malik et al. (65) have demonstrated that *S*-nitrosylation affects CLIC4 nuclear translocation, enhancing the association of the channel protein with two proteins responsible for the nuclear import, i.e., importin α and Ran, and thus increasing the nuclear levels of CLIC4. It has been proposed that through this mechanism CLIC4 would be able to induce apoptosis when NO cellular levels overcome the denitrosylating capability of the cell. Moreover, this would explain the mislocalization of CLIC4 in tumor cells where the redox balance is altered (65, 66).

S-nitrosylation can be responsible not only for the protein nuclear translocation but also for protein redistribution inside the cell. This is the case of the Tumor necrosis factor receptor superfamily member 6 (also known as Fas/APO-1/CD95), a cell surface receptor able to induce apoptosis when stimulated by the ligand FasL/CD95L or agonistic antibodies. After stimulation, the receptor recruits a number of proapoptotic factors - including caspase-8/10 and procaspase 8/10 to name a few—to assemble the death-inducing signaling complex (DISC) (67). Cancer cells evolved several ways to elude the possible apoptotic induction by CD95, for instance regulating the expression of the receptor (68) or inhibiting the interactions between the members of the DISC complex (69). A few years ago, it has been demonstrated that Fas undergoes *S*-nitrosylation at Cys199 in the transmembrane domain and Cys304 in the death domain, with both the events involved in determining Fas membrane localization, the latter also favoring Fas aggregation and translocation in plasma membrane lipid rafts (70, 71). As already mentioned, cancer cells can lose sensitivity to Fas-mediated apoptosis because of a decreased Fas expression, but *S*-nitrosylation (and inducers of this PTM) may be able to recover this sensitivity (70).

Transcription Factors in NO-Dependent Signaling

As mentioned above, the Ras family of proteins—that owes its fame to the fact of being very often mutated in most of the cancer types—is a known target of *S*-nitrosylation (see section Influence of *S*-nitrosylation on Catalytic Activity of Enzymes). To fully appreciate the regulation of Ras family members and Ras-dependent tumorigenesis, another player needs to be considered, adding an extra layer of complexity to an already intricate scenario. In 2012 Ras was shown to activate Ets-1 transcriptional activity in human ER-negative (ER-) breast tumors (72). Ets-1 is a proto-oncoprotein member of the Ets family of transcription factors sharing a unique DNA binding domain. Being expressed in a large variety of cellular types, Ets-1 has a role both in physiological and pathological conditions but its role in carcinogenesis is due to its ability to regulate the expression of angiogenic and extracellular matrix remodeling factors promoting an invasive phenotype (73, 74). When performing the promoter region analysis of genes up-regulated in ER- breast tumors showing high levels of NOS2 expression, Switzer et al. observed that the common denominator of the promoters of these genes was the presence of the Ets-binding sequence, pointing at the role of this transcription factor in the NOS2 (and thus NO)-dependent oncogenic signaling (72). In fact, Ets-1 activation following phosphorylation by MEK/ERK—in turn, activated by SNO-Ras—resulted in the expression of basal-like markers, as P-cadherin and S100A8 to name a few (75), as well as metastasis-related proteins such as CTSB and MMP-7 (76). Since breast tumors, differently from other cancer types, more rarely harbor Ras mutations (77), the *S*-nitrosylation of Ras and thus the activation of Ets1 signaling axis may indeed explain the wild-type Ras-mediated tumorigenesis of cancers overexpressing NOS2.

Protein Stability

S-nitrosylation can also directly affect protein stability and turnover in different ways, including cases in which protein stability and/or the propensity for proteasomal degradation is enhanced or reduced upon the *S*-nitrosylation of target cysteines.

An example is the caveolin-1 (Cav-1) protein that is enriched in 50–100 nm sized cell membrane invaginations with a structural role. These invaginations are implicated in many cellular pathways, such as endocytosis, lipid homeostasis, and signal transduction (78). Cav-1 has been described as having a controversial role in cancer development, with pro- and anti-tumorigenic effect depending on the context and the specific cancer type (79, 80). Interestingly, a relation between Cav-1 and NO has emerged in the context of anoikis - i.e., the detachment-induced apoptosis—in lung cancer cells, where it has been demonstrated that Cav-1 is rapidly ubiquitinated and degraded by the proteasome after cell detachment and anoikis (81). In the same work, the authors observed that, upon exposure of the cell to NO donors, *S*-nitrosylation of Cav-1 was able to stabilize the protein—inhibiting its proteasomal degradation—pointing at a

crucial role of the NO-mediated stabilization of Cav-1 in anoikis regulation.

Solid tumors are often characterized by a hypoxic (or even anoxic) microenvironment caused by scarce oxygen supply. Cancer cells activate a series of pathways promoting angiogenesis and other survival pathways to overcome hypoxia (82, 83). The master regulator of the activation response to low oxygen tensions is represented by the hypoxia-inducible factor 1 (HIF-1), a transcriptional complex made up of the constitutively expressed HIF-1β/ARNT and HIF-1α subunits, continuously synthesized, and degraded under normoxic conditions to be finally stabilized by hypoxic conditions (84). Because of this tangled role in cancer development, HIF-1 has been (and still is) at the heart of many scientific studies that have highlighted the existence of a multitude of post-transcriptional and post-translational mechanisms for regulating the activity of this protein (85). The degradation pathway of HIF-1α subunit in normoxic conditions starts with its hydroxylation by prolyl-hydroxylases (PHDs) in the oxygen-dependent degradation (ODD) domain. This event then targets the protein for ubiquitylation and the subsequent rapid degradation by the proteasome (86, 87). With regard to this, *S*-nitrosylation plays an active role in controlling the stability of the HIF-1α subunit. Indeed, in murine cancer cell line models, Li et al. (88) demonstrated that exposure to ionizing radiation stimulated NO generation in tumor-associated macrophages (TAMs). As a consequence, HIF-1α underwent *S*-nitrosylation on Cys533 (corresponding to the human Cys520), in turn inhibiting its binding with the E3 ubiquitin protein ligase part of the von Hippel-Lindau (vHL) tumor suppressor protein complex responsible of HIF-1 α degradation. This mechanism is of interest not only in relation to the already established role of HIF-1α in cancer therapy (89) but also for other diseases involving the immune system and inflammation (90).

We already mentioned the role of molecular chaperones, such as Hsp90, in cancer development and the regulation of its activity through *S*-nitrosylation (see section Influence of *S*-nitrosylation on Catalytic Activity of Enzymes). Hsp90 is highly conserved and with up to four different homologs in higher eukaryotes, such as the mitochondrial tumor necrosis factor receptor-associated protein 1 (TRAP1) (91). Recently, TRAP1 has been reported as a target of *S*-nitrosylation on its Cys501 with the effect of making the chaperone more prone to proteasomal degradation (40). In fact, levels of TRAP1 were reduced in human hepatocellular carcinoma cell lines depleted of *S*-nitrosoglutathione reductase (GSNOR), representing the best documented denitrosylase involved in regulating the levels of SNO-proteins in the cell (see section Enzymatic Regulation of *S*-nitrosylation). TRAP1 degradation was indicated being the causative event underlying the increase of succinate dehydrogenase (SDH) levels and activity. A few years ago it has been shown that TRAP1 is able to support cancer growth decreasing SDH activity, this leading to HIF-1α stabilization through the increase of succinate levels (41).

Recently, we and others showed that structural computational methods efficiently predict when mutations are likely to destabilize the protein in the context of disease-related mutations (including cancer) (92–95). Our measurements correlate with the rate of proteasomal degradation (95). In principle, similar approaches could be applied to predict how *S*-nitrosylation of Cys could alter protein stability and proteasomal degradation, as well as if the effect is likely to be structurally destabilizing or not. A major bottleneck in this context is the current lack of proper parameters for this uncanonical modification (see section Physical Models for *S*-nitrosothiols). Another computational approach useful to this scope could be the estimate of solvent accessibility of the ubiquitination sites for proteasomal degradation of the target proteins on structural ensembles collected by molecular simulations of *S*-nitrosylated and unmodified protein structures. A SNO-induced propensity to faster rates of proteasomal degradation might be also associated with higher solvent accessibility of the ubiquitination sites induced by the PTM.

Protein-Protein Interactions

An additional way used by *S*-nitrosylation to regulate protein function is through the control of protein-protein interactions. In the context of cancer progression, integrins represent a family of cell adhesion receptors that are attractive targets due to their ability to regulate cell morphology, cell-cell interaction, and signal transduction in the extracellular matrix (ECM). These processes are potentially linked to all the stages of tumor development (initiation, progression and, metastasis) (96). In 2012, Isaac et al. have demonstrated that in prostate cancer (PCs) cell lines several cysteines of integrin α6 (ITGα6)—a subunit of integrin α6β1, usually overexpressed in PC cells and the corresponding lymph node metastases - are targets of *S*-nitrosylation. Particularly, Cys86 *S*-nitrosylation of ITGα6 enhances its binding to ITGβ1 (overexpressed in PC cells too), decreasing the extent of cell adhesion and potentially explaining the ability of iNOS and NO to promote migration of the cancer cells (97, 98).

In conclusion, despite the fact that we illustrated separately the different strategies used by *S*-nitrosylation to impact on the cellular proteome, it is important to bear in mind that many of the processes individually discussed are however highly interconnected, as well as they can occur for the same SNO target. The relation between *S*-nitrosylation and cancer can be seen as a tangled map of connections, many of which are still poorly defined.

PREDICTION AND ANNOTATION OF *S*-NITROSYLATED PROTEINS

The Database of *S*-nitrosylated Proteins: dbSNO

Technical advances in mass spectrometry-based proteomics have improved the identification of *S*-nitrosylation sites (14, 99–103) and contributed overcoming the challenges due to the labile and highly dynamic nature of the thiol redox forms. This has been made possible by the availability of chemically-selective approaches to detect thiol redox modifications in concert with mass spectrometry-based proteomics (103).

These advances made now available a large list of experimentally identified SNO sites that require proper annotations and curations in publicly available repositories.

dbSNO2.0 (http://dbSNO.mbc.nctu.edu.tw) has been developed as a freely available resource to collect and explore the SNO sites of experimentally-identified *S*-nitrosylated proteins (104, 105). Moreover, regulatory networks are annotated for the *S*-nitrosylated proteins in the database, along with annotation of relevance in disease. dbSNO is the first manually curated repository for SNO peptides accounting for more than 4,000 SNO sites in almost 3,000 proteins. Annotation on targets with known 3D structure are also included together with information on solvent accessibility, neighboring residues and side-chain orientation for up to 298 substrate cysteine residues.

dbSNO2.0 can provide a useful framework for the redox biology community thanks to its availability of structural annotation for the computational and modeling studies on *S*-nitrosylated proteins. The bottleneck will be a regular update and curation of this research. We could expect that the number of characterized SNO sites will grow at a fast rate if we consider that the computational analyses of annotated protein databases using predicted SNO motifs indicate that up to 70% of the proteome may be targeted by *S*-nitrosylation (see section Prediction of *S*-nitrosylated Targets and SNO Sites). In the future, the SNO community could benefit of more collaborative and interdisciplinary efforts to develop and maintain a broader up-to-date repository and where also the experimental datasets from SNO-proteomics can be deposited for re-analysis, inspired by the efforts that the genomic community already established.

Prediction of *S*-nitrosylated Targets and SNO Sites

In the last few years, as the topic of *S*-nitrosylation gained importance and recognition among researchers, several attempts have been made to predict *S*-nitrosylation sites on proteins based on experimental data as a reference, using machine-learning algorithms on protein sequence data. Nonetheless, predicting SNO-sites has proven challenging for a number of reasons. First of all, a simple sequence linear motif that clearly identifies *S*-nitrosylation sites does not exist, as detailed in section Computational Structural and Chemical Studies of *S*-nitrosylation. The chemical-physical features of the environment surrounding the SNO site are crucial determinants for SNO specificity, and the pK_a of the thiol moiety of a cysteine greatly influences its propensity to be *S*-nitrosylated. Accurate methods for the prediction of pK_a from the 3D structure of proteins are currently available, such as PROPKA (106). Thus, the prediction of cysteine pK_a would be in principle a viable alternative when the 3D structure is available, or, even better, when an ensemble is available and protein flexibility can be taken into account (107). However, an analysis of structural determinants for *S*-nitrosylation showed that, at least in a limited data set of 55 proteins, features such as the acid–base motif flanking NO-Cys, hydrophobic content, predicted pK_a and solvent accessibility do not distinguish SNO-sites from non-*S*-nitrosylable cysteines (108). This study also reports that the presence of a SNO-site is correlated with the presence in the surrounding area of an acid-base motif, possibly with different functions than the mere activation of Cys or stabilization of SNO, such as in facilitating protein-protein interactions that would, in turn, induce *S*-nitrosylation. These results indicate that, while the local chemical environment around the SNO sites is certainly influencing reaction rates, predicting *S*-nitrosylation could benefit from a less reductionist approach.

Despite what just described, machine learning approaches tested so far have used protein sequences and sequence-derived features only; furthermore, even considering these, the set of experimental data on which machine algorithms can be trained is limited, at least respect to other PTMs such as phosphorylation, and typically unbalanced. This problem has been partially relieved by the publication of curated collections of SNO sites, such as the dbSNO database (105) and other manually curated datasets, as for example those by Xue et al. (109) and Li et al. (110). These resources do not alleviate the issue of using sequence data exclusively. Approaches based on protein structure would probably be more viable or at least include features that are more relevant to the problem at hand.

Given the limitations above, it is not surprising that the performance of SNO-site predictors has been so far consistently lower than predictors for other types of PTMs, with a Matthews correlation coefficient typically between 0.2 and 0.4. It should be emphasized, however, that no systematic review of performance on a common and independent test set has been performed so far. The fact that some of the methods have not been released as implementation or source code and that some of the web servers for SNO-site prediction are unreachable at the time of writing also makes fair comparisons difficult.

The different approaches tested so far for the prediction of SNO-sites are summarized in **Table 1**. Most of them relied on machine learning algorithms such as Support Vector Machines or (SVM) k-Nearest Neighbours (kNN) to classify Cys residues as *S*-nitrosylation sites or not. As feature space, all the methods considered amino-acid composition of the residues flanking the putative SNO-cysteine, often together with more structurally-oriented predictions such as physicochemical properties of amino acids, predicted secondary structure, predicted disorder propensity. This led, in turn, to a relatively high number of features among which the most discriminating and uncorrelated ones are selected, ranking them using methods as the minimal redundancy maximal relevance (mRMR) or the relative entropy selection. From a historical perspective, computational prediction of SNO sites has been tackled since 2006, when Hao et al. trained a support vector machines (SVM) classifier on a small dataset of SNO-proteins experimental data. The model turned out to barely outperform random chance (111). The first released algorithm designed to classify cysteine residues is named GPS-SNO (Group-based prediction system) (109), which uses an optimized scoring function to discriminate between SNO and not-SNO sites. Another prediction tool named SNOSite (112) was released in 2011, based on a collection of SVM models, each derived from sequences clustered according to a maximal dependence decomposition scheme to obtain statistically significant conserved motifs. CPR-SNO used a SVM

TABLE 1 | Summary of current methods for prediction of *S*-nitrosylation.

Name	Year	Availability	Link	Number of citations (Google scholar, April 2018)	PMID
GPS-SNO	2010	Webserver, standalone	http://sno.biocuckoo.org/	111	20585580
SNOSite	2011	Webserver	http://csb.cse.yzu.edu.tw/SNOSite/Prediction.html	54	21789187
CPR-SNO	2011	Webserver	http://math.cau.edu.cn/CPR-SNO/CPR-SNO.html[a]	21	21271979
–	2012	None	None	54	22178444
iSNO-PseAAC	2013	Webserver	http://app.aporc.org/iSNO-PseAAC/	112	23409062
iSNO-AAPair	2013	Webserver	http://app.aporc.org/iSNO-AAPair/	113	24109555
PSNO	2014	Webserver	http://59.73.198.144:8088/PSNO[a]	42	24968264
–	2014	None	None	5	25184139
iSNO-ANBPB	2014	None	None	41	24918295

[a]*Link to the website not working at the time we wrote this review article.*

classifier in which different encoding schemes for the protein sequence flanking the potential SNO-Cys were tested (113). In 2012, Li et al. (110) used the minimal-redundancy maximal-relevance (mRMR) method to determine the importance of several features of the sequence flanking C-sites to predict SNO-sites. The features included amino acid type, physicochemical features and conservation, disorder and secondary-structure propensity as well as solvent accessibility. Incremental feature selection was used to determine the set of features that would give the best performance. In 2013, two different predictors by Xu and colleagues were also released. The first, iSNO-PseACC (114) uses position-specific amino acid propensity into the form of pseudo amino acid composition with conditional random field models to predict the presence of SNO-sites, while the second, iSNO-AAPair (115) takes into account the coupling effects between residues close in sequence. In 2014, a paper published by Zhang et al. (116) used a scheme similar to Li et al. (110), in which a subset of features was calculated from the flanking sequence, feature selection was operated on them using relative entropy selection and incremental feature selection, while classification was done through the kNN algorithm. The final model was made available through the PSNO web server. In 2014 again, a paper by Huang et al. (117) used a similar approach to Li et al. (110) which also uses the same feature set, using the kernel sparse representation classification together with mRMR. Finally, another method has been implemented in iSNO-ANBPB and released in 2014, which uses an adapted normal distribution bi-profile Bayes (ANBPB) for feature extraction together with SVM (118).

COMPUTATIONAL STRUCTURAL AND CHEMICAL STUDIES OF *S*-NITROSYLATION

S-nitrosylation Sites Specificity

Many mechanisms can lead to the *S*-nitrosylation of proteins: reaction with NO_2, S-transnitrosylation, thyil radical recombination, and transition metal catalyzed pathways (see also sections *S*-nitrosylation of Cysteine Residues and Enzymatic Regulation of *S*-nitrosylation). These different pathways do not necessarily take place in the same cellular and protein environment. Thus, it is especially difficult to describe the microenvironment properties driving the specificity of *S*-nitrosylation sites (4, 14, 119, 120).

Albeit no systematic environment features have been found to be predictive descriptors for this PTM, the structural microenvironment would favor the susceptibility of cysteines to react with NO or undergo *S*-transnitrosylation. However, one has to keep in mind that these features could also favor cysteine reactivity toward other PTMs, hence it is really difficult to depict a precise panel of conditions that have to be fulfilled for *S*-nitrosylation to take place (4, 119).

Intensive investigations on the subject led to the conclusion that several environment features might have an influence on the specificity of cysteine targeting mechanisms associated to *S*-nitrosylation. The latter are certain characteristics that are subtler than a simple primary sequence effect, so that no discernable consensus sequence to accurately predict Cys SNO sites has been found so far (14, 28, 111, 121). In the 90's, Stamler and coworkers were the first to report the role of basic and acidic side-chains in the proximity of a cysteine in promoting *S*-nitrosylation, highlighted by their work on the *S*-nitrosylated hemoglobin protein (122, 123). In Stamler's model, a histidine residue proximal to the Cysβ93 in the oxygenated R-state of hemoglobin facilitates a base-catalyzed *S*-nitrosylation whereas a proximal aspartic acid in the deoxygenated T-state favors acid-catalyzed denitrosylation, coupling the oxygenation status of the hemoglobin to the SNO formation and release during the respiratory cycle. In another work, the acid-base motif in SNO-proteins has been expanded to include residues −6 and +6 from the target Cys and the notion of hydrophobic regions surrounding the SNO-Cys (121).

Acid-base motifs have been also reported at the tertiary structure level, i.e., not as short-linear motifs in the sequence space. In this case, acidic and basic residues in the proximity of the SNO site are contributed by different regions of the protein that are brought together in the 3D structure, as first found in the methionine adenosyltransferase (MAT) protein (124). The *S*-nitrosylation of the Cys121 of MAT is indeed promoted by two

arginines which are distal in the primary sequence but close in the tertiary structure and lower the Cys pKa. Similarly, 3D acid-base motifs have been found in the case of caspase-3 and aquaporin-1 proteins (12).

Since these first works on hemoglobin and MAT, several data consolidated the hypothesis of a 3D acid-base motif (12, 108, 125–129). Overall, the charged acidic and basic side chains in proximity of the SNO site regulate *S*-nitrosylation by influencing both thiol pKa (and as a consequence their reactivity) and SNO stability. As a general rule, the presence of an acid-base motif within 8Å of the target cysteine could facilitate interactions that make it to *S*-nitrosylation—e.g., protein-protein interactions in the case of *S*-transnitrosylation (130–132).

Besides the acid-base motif, other structural characteristics appeared to drive the specificity of cysteine reactivity toward CysNO. For instance, the presence of hydrophobic residues in the surroundings of the *S*-nitrosylation sites has also been proposed. As a matter of fact, *S*-transnitrosylation and NO oxidation reactions may preferentially take place on cysteine residues located in a hydrophobic pocket because of their low pK_a that, in turn, make them prone to react (10, 131, 133–138). Hydrophobic environments favoring *S*-nitrosylation are not limited to hydrophobic residues in the protein target. Indeed, they can also be contributed by membranes or interactions with other proteins (12).

This specific environment may also favor interactions with transition metal nitrosyls (127, 128, 139), as well as stabilize radical nitrosating species in the case of thiyl radical recombination (128, 140). Interestingly, studies suggested that acid-base motifs would be correlated with location of the target cysteines on α-helices, whereas hydrophobic environments would be more associated with *S*-nitrosylation on Cys sites located in β-sheets (130). Further factors that govern *S*-nitrosylation specificity are steric hindrance and solvent accessibility, that have also been proven to be key-features for *S*-nitrosylation promotion (3, 127). These two determinants are especially important in the case of *S*-transnitrosylation, in which protein-protein interactions are crucial. However, bioinformatic investigations revealed that, surprisingly, around 50% of the *S*-nitrosylation and *S*-glutathionylation sites are actually deeply buried in the native structure of the protein (141). Several studies revealed that buried cysteines may still be susceptible to undergo *S*-nitrosylation when surrounded by hydrophobic residues, by channeling of NO to target thiols (6, 10, 131, 133–138). This might be the case of the recent buried SNO site discovered in c-Src kinase where *S*-nitrosylation has a clear effect on protein activity and relevance in a cancer context (see section Computational Structural and Chemical Studies of *S*-nitrosylation). NO oxidation might also be favored upon co-localization of the target protein with NOS isoforms (3, 119), indeed enhancement of *S*-nitrosylation has been observed especially in the presence of surrounding eNOS in the Golgi apparatus (17, 142, 143). Compartmentalization of NO is induced by its quick consumption after production (<0.1 s) through reaction with molecules that colocalize with NOS (17). Furthermore, several NOS and NOS-interacting proteins are well-known to undergo *S*-nitrosylation—e.g., S100A8/A9, Dlg-4 and Cav-1. Thus, co-compartmentalization of the target protein with NOS could be considered as one of the many factors driving *S*-nitrosylation specificity, with the nitrosyl group being transported to a distant cellular zone through *S*-transnitrosylation.

In the analysis of cysteine SNO sites, we should also take into account the capability of the cysteine side chain to be involved in hydrogen bonds and how the substitution of the SH group with SNO can affect the native hydrogen bond network. Cys can serve as a hydrogen bond (HB) donor when protonated (SH) as well as a HB acceptor in both protonated and deprotonated states (S^-) (144).

Hence, acid-base motif and hydrophobic pocket proximity, appropriate redox potential, solvent accessibility, steric hindrance and colocalization with NOS have been highlighted as significant factors driving *S*-nitrosylation site specificity. Based on these considerations, machine learning techniques have been used in order to investigate *S*-nitrosylation sites. Several web servers have been designed to predict cysteines susceptible to be *S*-nitrosylated based on the protein sequence, and the dbSNO database is also available online to probe CysNO environment in PDB entries and predict potential SNO-proteins that may play a role in NO signal regulation in cancer cells - see details in section Prediction and Annotation of *S*-nitrosylated proteins.

SNO-Induced Long Range and Allosteric Effects

Little is known about the potential distal effects induced by *S*-nitrosylation. Nevertheless, allostery has been proposed to be an important factor for the promotion of *S*-nitrosylation/denitrosylation (128), and allosteric effects due to *S*-nitrosylation have been observed to play an important role in the regulation of the activity of several proteins (97, 145–147). Allosteric SNO-induced effects have been also postulated for the denitrosylase GSNOR, pointing out an intriguing feedback regulatory mechanism of NO signaling (see sections GSNOR System and GSNOR in Cancer).

Another striking example is the inactivation of the inducible nitric-oxide synthase (iNOS) upon auto-*S*-nitrosylation. NOSs (iNOS, eNOS, and nNOS) are well known to work as homodimers (148), with—at the interface between the two monomers—a zinc atom coordinated to four cysteines in a conserved ZnS_4 motif (149, 150). The latter is of utmost importance for the protein-protein interactions and the integrity of the homodimer, hence its activity. Therefore, the regulatory effects linked to the allosteric disruption of the interactions at the dimer interface have been extensively investigated for the design of new NOSs ZnS_4-related therapeutics (151–155). Interestingly, this site is specifically *S*-nitrosylated at the Cys109 position in iNOS. The latter induces the release of Zn^{2+}, coupled to a strong destabilization of the dimer, resulting in its disruption and iNOS inactivation (145). Upon high NO concentration, *S*-nitrosylation of iNOS limits its activity (i.e., NO production) through an allosteric mechanism driving the dimer/monomer balance (145, 156). Thus, the Cys109 specific *S*-nitrosylation plays an important role in the regulation of iNOS activity,

whose dysfunction has been shown to play an important role in tumor growth in several cancer types (157). Noteworthy, eNOS dimer dissociation upon treatment with NO donors has also been reported (158), suggesting a similar equivalent autoinhibitory mechanism. Similar structural impact, although less pronounced, has been reported for the NMDA receptor, for which *S*-nitrosylation has been suggested to allosterically regulate the ligand-binding, regulatory and linker regions (146, 159, 160). Likewise, the cyclic nucleotide-gated channels (CNGcs), a class of ionic channels that permeate cations and are especially important in sensory-receptor cells, might be allosterically regulated by *S*-nitrosylation of a specific cysteine located in their ligand-binding region (147).

Besides, relatively small distal effects of *S*-nitrosylation have been observed in certain X-ray structures of SNO-proteins (161–164). This behavior nicely fits in the context of allosteric effects that can occur without a marked change in protein shape, as attested in many other biological cases (33, 165). For instance, hemoglobin (Hb) *S*-nitrosylation leads to local reorganization but no large changes in the quaternary structure of the tetramer in the crystals (162). However, the authors suggested that larger effects might occur in solution, with a shift in the balance between Hb R and R2 states. In a similar fashion, S100A1 *S*-nitrosylation leads to distal reorganizations of the linker region and the two helices III and IV from the C-terminal EF-hand, that are known to be important for target recognition (164).

Nevertheless, the paucity of experimental structures of SNO-proteins does not allow to efficiently probe the distal effects that could be induced by *S*-nitrosylation. In this context, methods based on MD simulations using reliable force field parameters for Cys-NO would constitute a considerable asset to explore such reorganizations. Indeed, many methods to study distal conformational changes, including methods based on MD-derived ensemble, have been proposed and successfully applied to the study of long-range effects induced by protein PTMs such as phosphorylation or other perturbation of the protein native structure (32–35, 166–168) and they can be naturally translated to the study of allosteric effects promoted by *S*-nitrosylation.

A Case Study: Effects of *S*-nitrosylation on Src Kinases

Kinases are usual suspects in the context of cancer research (169–171). Intriguingly, *S*-nitrosylation has been recently pointed out as regulatory mechanisms for kinases (141, 172), which are enzymes with the main regulatory role for another PTM, i.e., phosphorylation, highlighting an interesting cross-talk between different PTM cellular signals.

As mentioned in the previous sections, Src kinases are a family of kinases responsible for cellular proliferation, differentiation and survival (172). Disregulation of Src kinases has been linked to different cancer types. They are multi-domain proteins, including four different domains of which one carries out is the catalytic activity. Two tyrosines (Tyr416 and 527) are regulated by phosphorylation and their phosphorylation is responsible of either activation or deactivation of the kinase, respectively.

Apart from phosphorylation, another layer of post-translational regulation—relying on *S*-nitrosylation—has been demonstrated for the c-Src kinase. The SNO site is the Cys498, which is one of the nine cysteines of the human Src kinase (47). c-Src is known to promote cancer cell invasion and metastasis and its *S*-nitrosylation enhances the protein activity but the structural mechanisms behind this have been poorly understood. Cys498 is also conserved in other kinases of the Src family, suggesting a common mechanism, as attested by the fact that the c-Yes kinase activation is also mediated by NO (47). These data overall link NO-dependent activation of c-Src to cancer cell invasion and metastasis but the structural and molecular details are still elusive.

In a recent computational work, Rando and coworkers applied different computational structural analyses accounting for electrostatic, steric and hydrophobic properties to compare the Cys498 selective SNO site with the other three c-Src cysteines that are not affected by *S*-nitrosylation. Their data pointed in the direction of a rather buried and highly nucleophilic Cys with a highly hydrophobic environment in which NO can be more prone to undergo decomposition into the electrophilic intermediates (173).

As a future direction, structural studies to assess conformational changes in the *S*-nitrosylated and non-*S*-nitrosylated protein could shed new light on the NO-mediated activation of c-Src and other similar kinases, as well as properly assess the accessibility of the SNO site in the native conformational ensemble of the protein. Also, in this context, the main limitation is related to the poor availability of force field parameters to describe SNO proteins.

Physical Models for *S*-nitrosothiols (RSNOs)

The impact of *S*-nitrosylation on protein structure, function and stability can be different from one protein to the other (see section Biological Mechanisms Promoted by *S*-nitrosylation). Therefore, one could not describe a systematic structural and reactivity behavior of *S*-nitrosylated proteins. There is a real need of investigations by both experimental and theoretical means, which represent a colossal yet of utmost importance work with the aim at gaining knowledge about the important phenomena driving RSNOs formation and reactivity.

S-nitrosothiols exhibit a highly complex chemistry, which represents a real challenge for theoretical studies. In the last decade, efforts have been dedicated to the development of an accurate structural and electronic description of the -SNO group, by both experimental and theoretical means (174–176). Concerning theoretical investigations, a certain amount of high-level quantum chemistry studies has been performed on small RSNOs (mainly with R=H,Me). The main part of the theoretical studies focused on the description of the thionitrous acid HSNO, the smallest RSNO (128, 143, 177–180). Indeed, the high complexity of the -SNO moiety requires the use of time-demanding calculations. Thus, the size of the system is rapidly limited by the computational resources. The nature of the S-N bond is especially challenging to investigate, and lots of efforts

have been made to gain information about the properties of this bond. Values of the bond dissociation energy (BDE) and the activation energy corresponding to the transition between the *cis* and *trans* isomers have been computed using diverse levels of theory (181–184). One of the most recent computational studies using high level CCSD(T) coupled to CBS extrapolation methods (179) suggested that the *cis* - *trans* interconversion requires an activation energy up to ∼9 kcal/mol, with the *cis* isomer slightly less stable than the *trans* one, roughly by 0.1 kcal/mol. Investigations on larger RSNOs moieties have shown that the nature of R may have a strong influence on RSNO chemistry, hence CysNO structure might exhibit different features than HSNO (128, 185–189). However, there is still a lack of theoretical studies about CysNO properties, with only few investigations being reported concerning models structurally closer to the *S*-nitrosylated cysteine than HSNO.

Electronic properties of the -SNO group have been highly investigated by computational means. Especially, a lot of work is available on HSNO structure description by high-level QM methods and even Car-Parrinello metadynamics (179, 181, 184, 190–192), which shed light on the multi-reference character of the -SNO group. Indeed, the complexity of the S-N bond nature might result from the combination of three different resonance structures: the neutral -S-N=O, the zwitterionic -S^+ =N-O^-, and the RS^-/NO^+ ion pair. Further investigations on the larger CH_3SNO model confirmed this feature, yet it seems less pronounced for CH_3SNO than HSNO (186). Unfortunately, no further evidences about RSNOs multi-reference character has been reported for larger models of RSNOs, the computational cost of such high-level calculations being prohibitive.

Nevertheless, these investigations underlined the difficulty to obtain an accurate description of *S*-nitrosothiols structure, with the S-N bond exhibiting very complex chemical properties. Hence, one should pay a particular attention while dealing with such systems, by starting with a careful choice of the level of theory.

The unusual electronic structure of RSNO and its multi-reference character makes it a difficult system to accurately model. Hence, high level *ab initio* QM methods should be used in order to obtain a reliable and accurate description of the complex electronic density of the SNO moiety. However, the use of such quantum calculations is computationally very demanding when it comes to study models larger than HSNO or CH_3SNO. Thus, benchmark studies have been led to assess the capacity of the less time-consuming DFT and TD-DFT methods to reproduce structural and electronic features obtained by high level *ab initio* methods and experiments. Overall, several of these theoretical investigations highlighted the reliability and robustness of the B3P86 functional with large basis sets for the calculation of S-N bond energy dissociation, spectroscopic and structural properties of small RSNO (182, 189, 193). Likewise, the PBE0 functional with large basis sets has also been revealed as a good compromise between computational time and accuracy in describing RSNO properties (179, 186, 188, 194). However, it is always strongly recommended to verify results obtained by DFT methods using more computationally demanding higher-level methods (186). Likewise, experimental data on RSNOs characteristics, mainly from NMR studies, have been reported and can validate values predicted by computational chemistry (44, 195–197).

The electronic and structural properties of RSNO are highly modulated by the molecular environment, especially in the presence of proximal charges (e.g., upon coordination to metal ions), with large fluctuations of the S-N bond stability being observed in several experimental and theoretical works (182, 185, 188, 198, 199). Studies of RSNO interacting with metals especially highlighted the dramatic influence of surroundings on the S-N bond nature. For instance, coordination to Cu^I, which is known to play important roles in NO-release regulation by catalyzing the decomposition of RSNOs, tends to weaken the S-N bond upon S-coordination while N-coordination was predicted to strengthen it (182, 200).

An interesting property of the -SNO moiety is the multi-reference character induced by the dramatic difference between its resonance structures (**Figure 4**), as highlighted in several theoretical works published by Timerghazin's group (184–186, 188, 201). Indeed, the use of an external electric field (EEF) in QM calculations brought out the high polarizability of the–SNO moiety, with modulation between two minor resonance structures, which exhibit opposite charge distribution and reactivity. The first one exhibits an ionic structure, with the charge located mainly on the sulfur atom, interacting with the electrophilic NO^+ moiety through a long and weak S–N bond. Upon opposite polarization of the EEF, the -SNO adopts a totally different configuration, with the charge located on the NO double bond, the sulfur atom being in this case prone to undergo nucleophilic attacks. The balance between

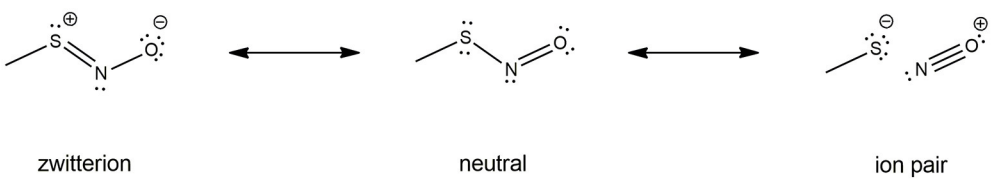


FIGURE 4 | RSNO resonance structures, balanced between the neutral **(center)**, the zwitterion **(left)** and the ion pair **(right)** forms. The *trans* conformer is depicted here, but the *cis* is also possible though less stable, as mentioned in section Physical Models for S-nitrosothiols (RSNOs). The neutral form is the most abundant one, the other ones being only minor conformations with dramatically opposite features—hence the dual reactivity of RSNOs with nucleophiles. The relative abundance of the three RSNO forms is highly depend on its microenvironment and the nature of the R group. For instance, the neutral/zwitterion/ion pair ratio is 79/11/10% vs. 75/15/10% for HSNO and CH_3SNO respectively.

the zwitterionic, neutral, or ionic conformations might thus be highly influenced by the electric field induced by the -SNO chemical environment and lead to a fluctuating reactivity of this moiety (185). The coexistence of such antagonist structures brings explanation to the contradictory observations that have been published concerning RSNO structure and reactivity (193, 202–205).

Nowadays the computational resources available do not allow to model the dynamics of an entire protein at the QM level of theory. Thus, investigations about the *S*-nitrosylated cysteine electronic structure have been performed on reduced models only. The largest one reported so far being the portion of an α-helix *S*-nitrosylated *in silico*, on which Talipov et al. performed QM/MM calculations to probe the effect of proximal charged amino acids on CysNO electronic structure (188). Nevertheless, QM/MM calculations are way too time-consuming to be systematically used to investigate influence of the protein environment in realistic models, since the dynamics of the system might also have a strong influence on the cysteine environment. Furthermore, Talipov et al. suggested that even small allosteric effects could induce dramatic effects on the CysNO reactivity. Thus, the dynamics of the system should be taken into account when studying the *S*-nitrosylated cysteine behavior in a protein environment. The use of all-atom MM-MD simulations can help toward this goal. However, attention should be devoted to the choice of the force field parameters used to describe the highly complex *S*-nitrosylated cysteine structure.

So far, AMBER and GROMOS force field parameters have been developed to describe CysNO and few case studies of proteins harboring CysNO by classical molecular dynamics simulations have been reported. On the one hand, the AMBER parameters developed by Han et al. have been generated using quantum mechanics (206) and validated using as a model system a *S*-nitrosylated thioredoxin crystal structure on Cys69 (207). On the other hand, Petrov et al. (208) parameterized CysNO by deriving GROMOS force field values. Moreover, online tools have been developed to insert PTMs *in silico* using GROMOS force field parameters, including the cysteine *S*-nitrosylation: the Vienna-PTM and the Automated Topology Builder (ATB) servers (209–211). Nevertheless, an in-depth look into the values of these two different sets of parameters highlights not negligible differences (**Tables 2**, **3**). Thus, these parameters have to be extensively validated against experimental data to assess their efficiency in describing a such complex chemical structure. The major bottleneck in this field is related to the fact that only a minority of experimental structures is available by NMR and X-Ray crystallography (161–164, 207, 212–216) and classical MD simulations using reliable force fields usually provide predictive data about structural and dynamical behavior of biosystems. However, in the CysNO case, it would be difficult to model with standard force fields using point charges, given that the polarization of the—SNO moiety is likely to undergo a dramatic variation of the reactivity depending on its micro-environment. To overcome this issue, one might consider to use polarizable force fields, which are undergoing marked improvements (217).

Overall, investigations that have been led so far on small RSNO models provide a solid basis toward the understanding of *S*-nitrosylated cysteines structural properties within a protein environment. The system size and the simulation time scale are known to be very rapidly prohibitive while using QM and hybrid QM/MM (-MD) methods. Besides, classical MD simulations are nowadays a method of choice for the theoretical investigation of dynamics of proteins and macro-molecules in general (32, 33, 218), but its reliability relies on the force field accuracy. Hence, a particular effort should be realized in order to bypass limitations of standard force fields and find a sustainable solution in order to unravel the complex mechanisms underlying proteins *S*-nitrosylation.

TABLE 2 | Comparison between GROMOS and AMBER parameters for *S*-nitrosylated cysteine with regards to atomic charges.

	GROMOS	AMBER
Atomic charges (S)	0.1	−0.0735
Atomic charges (N)	0.35	0.0355
Atomic charges (O)	−0.45	−0.1522

REACTIVITY OF *S*-NITROSOTHIOLS

The unusual structure of *S*-nitrosothiols (RSNOs), balancing between three different mesomeric forms, implies a complex chemistry of these moieties. The biological relevance of RSNOs relies on their important role in NO storage and transport, as well as their function as HNO donors *in vivo* (219–224). As RSNOs are relevant to a broad spectrum of diseases, ranging from asthma to cancer (6, 8, 140, 225–227), extensive works have been reported with the final goal of developing new SNO-related therapeutical strategies. Efforts have been made especially for the design of new RSNO-inspired NO-releasing biomaterials (228–230). In this framework, a deep knowledge of the mechanisms driving the RSNOs reactivity is of utmost importance for the design of therapeutics, with implications for the treatment of a large range of diseases. Theoretical investigations have been led in order to unveil the electronic mechanisms ruling RSNO denitrosylation. The latter can take place through several reaction pathways: *S*-transnitrosylation, disulfide bridges formation (*S*-thiolation), and homolytic cleavage of the S-N bond—see **Figure 5**.

RSNO-Thiol Interactions

Albeit the *S*-transnitrosylation and *S*-thiolation have been studied extensively by experimental means (22, 26, 28, 131, 231, 232), there is only a little amount of theoretical data available. A proper electronic description of the *S*-nitrosylated cysteine requires the use of high-level quantum methods, whereas the current computational resources do not allow to study such a reaction in a protein model, meaning that current studies are limited to small RSNO models. Moreover, only one work has been published about the electronic mechanisms driving *S*-thiolation by Ivanova et al. using MeSNO and MeSH models (194). According to their DFT calculations on this simplified model system, the reaction takes place in three phases. First, the thiol proton is transferred to the—SNO nitrogen atom,

TABLE 3 | Comparison between GROMOS and AMBER force field (FF) parameters for *S*-nitrosylated cysteine (i.e., bonds, angles, dihedral angles).

	Bonds			Angles		Dihedral angle
	C-S	**S-N**	**N-O**	**C-S-N**	**S-N-O**	**C-S-N-O**
FF NAME						
GROMOS	gb_31	gb_31	gb_5	ga_30	ga_26	gd_21
AMBER	CT SH	SH NC	NC O	CT SH NC	SH NC O	CT SH NC O
EQUILIBRIUM DISTANCE/ANGLE						
GROMOS	0.178	0.178	0.123	121	120	0
AMBER	0.181	0.1755	0.1165	103.96	116.58	180
FORCE CONSTANT (kJ mol^{-1} nm^{-1})						
GROMOS	5.94E+06	5.94E+06	1.66E+07	685	530	16.7
AMBER	1.98E+05	2.77E+05	6.61E+05	354.8	527.37	22.51
PHASE						
GROMOS	/	/	/	/	/	2
AMBER	/	/	/	/	/	2

leading to a thiolate MeS^- and the $MeSNHO^+$ moiety. The latter undergoes a strong delocalization of the electronic density on the oxygen atom, increasing the electrophilicity of the sulfur group. Meanwhile, the thiol sulfur moves out of plane to initiate the nucleophilic attack on the -SNO sulfur atom. The second phase is the formation of the S-S bond, which results in a zwitterionic species involving a tri-coordinated sulfur. The third and last phase consists in the release of HNO following the S-N bond cleavage. A similar reaction mechanism has been proposed by Moran et al. (233) for the hydrolysis of O-protonated RSNO.

The addition of explicit water molecules in the model system highlighted the possibility of a reaction assistance, with an activation energy decrease up to 20 kcal/mol. The water molecules participate to the proton transfer, but also have a stabilizing effect on the charge-separated intermediates. This stabilization is even more pronounced when moving from gas phase to a polarizable aqueous environment by using an implicit solvent model, suggesting that the environment provided by the protein embedding might also offer a similar stabilization. This hypothesis is supported by investigations highlighting the effect of external electric fields on the RSNO electronic density, that can lead to its dramatic polarization (185, 186, 188). Noteworthy, the biologically relevant competition between the *S*-thiolation and the *S*-transnitrosylation processes might then be driven by the polarization of the -SNO by its surroundings. The ionic conformation would favor thiolate attack on the NO^+ (i.e., *S*-transnitrosylation) while its antagonist structure might be prone to undergo nucleophilic attack of thiolate on the sulfur atom with release of a HNO molecule—i.e., *S*-thiolation and disulfide bridges formation (188). Very recently, a work by Wolhuter et al. suggested that *S*-nitrosothiols could be reactive intermediates leading to disulfide bridges formation and *S*-thiolation (234). Their work highlighted the strong propensity of *S*-nitrosothiols to induce disulfide bond formation, pointing out the larger instability of *S*-nitrosothiols compared to disulfides. According to their results, *S*-nitrosothiols might be only transient moieties favoring the formation of S-S bonds, rather than the putative stable redox regulators. The study of cysteines nitrosothiols reactivity, involved in the promotion of disulfide bridges formation, is of major importance for the understanding of the complex mechanisms ruling the cell redox regulation. We thus envision that further theoretical and experimental efforts are still necessary to strengthen our knowledge in this area.

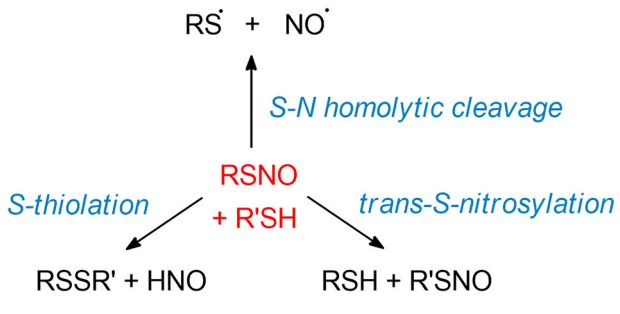


FIGURE 5 | SNO reactivity. We here illustrated the main reactions involving RSNOs. **Top:** homolytic cleavage of the S-N bond, consisting in the loss of nitric oxide with a remaining thyil radical species. **Left:** *S*-thiolation between RSNO and a R'SH thiol, leading to the formation of a disulfide bridge RSSR' and a nitroxyl moiety HNO. **Right:** *S*-transnitrosylation process inducing the transfer of NO from RSNO to a R'SH thiol. The detailed mechanisms of these reactions are still poorly understood and thus not depicted here.

Homolytic Cleavage of RSNO

Theoretical and experimental investigations about the *S*-nitrosothiols S-N bond dissociation stressed out the ease of this reaction, both thermal, and photochemical decomposition happening at room temperature (199, 235–240). The latter reaction induces the homolytic loss of NO, eventually leading to the formation of a stable S-S bond, which involves the reaction between two newly formed thyil moieties. The weakness of the S-N bond (values from 15 to 35 kcal/mol) (184, 186, 189, 193,

203, 219, 241) may favor its rapid decomposition, although the *S*-nitrosothiol chemical environment can strongly enhance its half-life (198, 199, 204).

Recent theoretical work about the S-N photo-dissociation in model compounds (small RSNOs) shed light on the barrierless character of the process upon irradiation in the visible and UV regions as well as upon exogenous photosensitization, with population of S_1, S_2, and T_1 states respectively (189). Experimental investigations showed that irradiation of *S*-nitrosothiols at UV-visible wavelengths induces the release of nitric oxide and a thyil radical (236, 242). Likewise, photo-decomposition of RSNOs has been observed in human skin upon UVA irradiation (243). Similar wavelengths have been reported for light-induced release of NO from NO-metal complexes (mostly iron, but also Mb, Co, or Ru among others), which has been widely investigated for its biological relevance and has been observed in the UV-vis-NIR regions depending on the nature of the complex (244–247).

Thermal decomposition of small RSNOs has also been investigated using high-level computational methods (184, 186, 201). In the most recent of them, Khomyakov and Timerghazin underlined the difficulty to obtain accurate values of the S-N bond dissociation energy, the convergence of the S-N bond features being especially slow due to the unusual, multi-reference character of the -SNO moiety (186). Their calculations suggested a value of 32.2 kcal/mol for the CH_3SNO model S-N bond cleavage in implicit aqueous solvent, with a very small stabilization of energy when shifting from water to the non-polar diethylether solvent—often used to mimic the protein environment. Noteworthy, metal ions and especially copper ions can efficiently catalyze the S-N bond thermo-dissociation, making it an ultrafast process (182, 236, 248, 249).

NO transport and storage are mainly provided by iron-nitrosyl (e.g., in myoglobin and hemoglobin) and nitrosothiols compounds. These processes are known to be of utmost importance for the modulation of important cellular mechanisms such as the mitochondrial respiration (250) or vasodilation/cardioprotection (251). As mentioned previously in this review, nitric oxide is also known to have a multifaceted role in cancer biology, with both tumor-suppressing and tumor-promoting effects reported (252, 253). The redox chemistry of *S*-nitrosothiols is as rich as complex and is related to a large variety of biochemical processes. It is notably balanced by pH, temperature, UV-vis irradiations, RSNO micro-environment (influencing its pK_a), chemical nature of RSNO, and presence of reactive compounds such as metal ions (254, 255). The dysregulation of NO flux in the tumor micro-environment has also been found to modulate the redox signaling pathways during cancer progression (256), which might consequently influence RSNOs reactivity. Several compounds are known to react with *S*-nitrosothiols such as phosphine derivatives, sulfenic acids, and a large variety of nucleophiles. Some of them have biological relevance (e.g., thiols and seleno compounds), but an important aspect of this broad reactivity is to provide perspectives for the development of efficient RSNOs detection methods and RSNO-based therapies—e.g., biotin labeling, phosphine compounds, and metal complexes (257–261). Noteworthy, low-molecular weight *S*-nitrosothiols derivatives such as *S*-nitroso-N-acetyl penicillamine (SNAP), GSNO and L-/D-CysNO are commonly used in biological experiments. For an in-depth description of RSNOs chemistry, very good articles, and reviews are available in the literature (254, 262, 263).

Considering their high biological relevance, mechanisms driving RSNO chemistry are matter of intensive investigations, and the ease of RSNOs dissociation through several pathways at room temperature, leading to the efficient release of nitric oxide, is an interesting property very often used in studies aiming at developing therapies against cancer and other diseases (264–271).

CONCLUSIONS

The discovery of *S*-nitrosylation opened new venues in the context of the cellular signaling induced by post-translational modifications since NO relies on this modification to transmit its redox signaling. The mechanisms involved in its regulation and the effects caused by it are very complex and diverse, as described above. *S*-nitrosylation has been emerging also as a key mechanism in many diseases, such as cancer. As a result of extensive efforts by cellular and proteomics studies, we now know several enzymatic regulators of *S*-nitrosylation, as well as a myriad of protein targets that are modulated by this post-translational modification. However, structural studies that can help in understanding the mechanisms induced by this modification and its reactivity are still not as in-depth as the investigations carried out so far for other more conventional PTMs, such as phosphorylation. The complexity of this redox modification challenges experimental and computational structural and biophysical studies. Nevertheless, advances in computational biochemistry hold promise to both generate new mechanistic hypotheses that can be experimentally tested and rationalize at the molecular and atom level the experimental results collected so far. Several efforts are still required to experimentally solve new structures of *S*-nitrosylated proteins, using techniques such as X-ray crystallography and NMR, as well as to collect experimental data probes the conformational changes induced by this PTM on both structure and dynamics at local and distal sites of the proteins. Once more information will be available, we will be capable of overcoming the limitations of standard force fields to model the effects induced by *S*-nitrosylation on protein structure and dynamics, as well as to unveil the long-range allosteric effects triggered by this redox PTM. Similar studies can, for example, shed new light in the context of identifying and characterizing SNO sites that are buried in the native structure and that can become available for modification upon transient conformational changes of the protein. Moreover, in the context of reactivity, theoretical studies will need to account for more complete models that can account for the structural environment of the *S*-nitrosylated cysteine. Prediction algorithms for SNO-sites would also largely benefit from more available experimental and structural data, hopefully making the current predictions more accurate. The redox community should also dedicate more comprehensive, collaborative, and organized efforts toward the development of a

common publicly available repository for the *S*-nitrosyloma with both sequence, structural and experimental information.

AUTHOR CONTRIBUTIONS

ML contributed to the writing of chapter 3, MA to the writing of chapter 4, EB contributed to the writing of chapter 6 and 7 and MT to the writing of chapter 5. EP wrote the introduction, chapter 2, conclusions and contributed to the writing of all the other chapters. All the authors revised the final version of the manuscript and contributed with figures and tables.

FUNDING

Our research in the context of redox modifications of protein is supported by an Alfred Benzon Principal Investigator Fellowship 2017–2019 to EP and EB, a PRACE-DECI-13th grant CHARPEDO (for computational access to the UK HPC infrastructure ARCHER), an ISCRA-LISA HPC grant RedoxC-HPL13PEJU2 (for calculations on Marconi) and a DeiC Pilot grant 2015–2016 (for calculations on the Danish HPC Infrastructure Computerome).

REFERENCES

1. Go YM, Chandler JD, Jones DP. The cysteine proteome. *Free Radic Biol Med.* (2015) 84:227–45. doi: 10.1016/j.freeradbiomed.2015.03.022
2. Fra A, Yoboue ED, Sitia R. Cysteines as redox molecular switches and targets of disease. *Front Mol Neurosci.* (2017) 10:167. doi: 10.3389/fnmol.2017.00167
3. Furuta S. Basal S-Nitrosylation is the guardian of tissue homeostasis. *Trends Cancer* (2017) 3:744–8. doi: 10.1016/j.trecan.2017.09.003
4. Stomberski CT, Hess DT, Stamler JS. Protein S-Nitrosylation: determinants of specificity and enzymatic regulation of s-nitrosothiol-based signaling. *Antioxid Redox Signal.* (2018) doi: 10.1089/ars.2017.7403
5. Stamler JS, Lamas S, Fang FC. Nitrosylation: the prototypic redox-based signaling mechanism. *Cell* (2001) 106:675–83. doi: 10.1016/S0092-8674(01)00495-0
6. Anand P, Stamler JS. Enzymatic mechanisms regulating protein s-nitrosylation: implications in health and disease. *J Mol Med.* (2012) 90:233–44. doi: 10.1007/s00109-012-0878-z
7. Martínez-Ruiz A, Lamas S. S-nitrosylation: A potential new paradigm in signal transduction. *Cardiovasc Res.* (2004) 62:43–52. doi: 10.1016/j.cardiores.2004.01.013
8. Bettaieb A, Plenchette S, Paul C, Laurens V, Romagny S, Jeannin J. *Nitric Oxide and Cancer: Pathogenesis and Therapy* . (Springer International Publishing) (2015).
9. Nakamura T, Tu S, Akhtar M, Sunico C, Okamoto SI, Lipton S. Aberrant Protein S-nitrosylation in neurodegenerative diseases. *Neuron* Switzerland: Springer International Publishing (2013) 78:596–614. doi: 10.1016/j.neuron.2013.05.005
10. Derakhshan B, Hao G, Gross SS. Balancing reactivity against selectivity: the evolution of protein S-nitrosylation as an effector of cell signaling by nitric oxide. *Cardiovasc Res.* (2007) 75:210–9. doi: 10.1016/j.cardiores.2007.04.023
11. Kovacs I, Lindermayr C. Nitric oxide-based protein modification: formation and site-specificity of protein S-nitrosylation. *Front Plant Sci.* (2013) 4:1–10. doi: 10.3389/fpls.2013.00137
12. Hess DT, Matsumoto A, Nudelman R, Stamler JS. S-nitrosylation: spectrum and specificity. *Nat Cell Biol.* (2001) 3:3–5. doi: 10.1038/35055152
13. Seth D, Stamler JS. The SNO-proteome: causation and classifications. *Curr Opin Chem Biol.* (2011) 15:129–36. doi: 10.1016/j.cbpa.2010.10.012
14. Gould NS, Evans P, Martínez-Acedo P, Marino SM, Gladyshev VN, Carroll KS et al., Site-specific proteomic mapping identifies selectively modified regulatory cysteine residues in functionally distinct protein networks. *Chem Biol.* (2015) 22:965–75. doi: 10.1016/j.chembiol.2015.06.010
15. Fang M, Jaffrey SR, Sawa A, Ye K, Luo X, Snyder SH. Dexras1: a G protein specifically coupled to neuronal nitric oxide synthase via CAPON. *Neuron* (2000) 28:183–93. doi: 10.1016/S0896-6273(00)00095-7
16. Villanueva C, Giulivi C. Subcellular and cellular locations of nitric oxide synthase isoforms as determinants of health and disease. *Free Radic Biol Med.* (2010) 49:307–16. doi: 10.1016/j.freeradbiomed.2010.04.004
17. Iwakiri Y, Satoh A, Chatterjee S, Toomre DK, Chalouni CM, Fulton D, et al. Nitric oxide synthase generates nitric oxide locally to regulate compartmentalized protein S-nitrosylation and protein trafficking. *Proc Natl Acad Sci USA.* (2006) 103:19777–82. doi: 10.1073/pnas.0605907103
18. Benhar M, Forrester MT, Stamler JS. Protein denitrosylation: enzymatic mechanisms and cellular functions. *Nat Rev Mol Cell Biol.* (2009) 10:1–12. doi: 10.1038/nrm2764
19. Collet J-F, Messens J. Structure, function, and mechanism of thioredoxin proteins. *Antioxid Redox Signal.* (2010) 13:1205–16. doi: 10.1089/ars.2010.3114
20. Lillig CH, Holmgren A. Thioredoxin and related molecules–from biology to health and disease. *Antioxid Redox Signal.* (2007) 9:25–47. doi: 10.1089/ars.2007.9.25
21. Sengupta R, Ryter SW, Zuckerbraun BS, Tzeng E, Billiar TR, Stoyanovsky DA. Thioredoxin catalyzes the denitrosation of low-molecular mass and protein S-nitrosothiols. *Biochemistry* (2007) 46:8472–83. doi: 10.1021/bi700449x
22. Benhar M, Forrester MT, Hess DT, Stamler JS. Regulated protein denitrosylation by cytosolic and mitochondrial thioredoxins. *Science* (2008) 320:1050–4. doi: 10.1126/science.1158265
23. Ben-Lulu S, Ziv T, Admon A, Weisman-Shomer P, Benhar M. A Substrate trapping approach identifies proteins regulated by reversible S-nitrosylation. *Mol Cell Proteomics* (2014) 13:2573–83. doi: 10.1074/mcp.M114.038166
24. Benhar M. Nitric oxide and the thioredoxin system: a complex interplay in redox regulation. *Biochim Biophys Acta* (2015) 1850:2476–84. doi: 10.1016/j.bbagen.2015.09.010
25. Forrester MT, Seth D, Hausladen A, Eyler CE, Foster MW, Matsumoto A, et al. Thioredoxin-interacting protein (Txnip) is a feedback regulator of S-nitrosylation. *J Biol Chem.* (2009) 284:36160–6. doi: 10.1074/jbc.M109.057729
26. Mitchell DA, Marletta MA. Thioredoxin catalyzes the S-nitrosation of the caspase-3 active site cysteine. *Nat Chem Biol.* (2005) 1:154–8. doi: 10.1038/nchembio720
27. Mitchell DA, Morton SU, Fernhoff NB, Marletta MA. Thioredoxin is required for S-nitrosation of procaspase-3 and the inhibition of apoptosis in Jurkat cells. *Proc Natl Acad Sci USA.* (2007) 104:11609–14. doi: 10.1073/pnas.0704898104
28. Wu C, Liu T, Chen W, Oka S, Fu C, Jain MR, et al. Redox regulatory mechanism of transnitrosylation by thioredoxin. *Mol Cell Proteomics* (2010) 9:2262–75. doi: 10.1074/mcp.M110.000034
29. Staab CA, Hellgren M, Höög J-O. Medium- and short-chain dehydrogenase/reductase gene and protein families : dual functions of alcohol dehydrogenase 3: implications with focus on formaldehyde dehydrogenase and S-nitrosoglutathione reductase activities. *Cell Mol Life Sci.* (2008) 65:3950–60. doi: 10.1007/s00018-008-8592-2
30. Yang ZN, Bosron WF, Hurley TD. Structure of human chi chi alcohol dehydrogenase: a glutathione-dependent formaldehyde dehydrogenase. *J Mol Biol.* (1997) 265:330–43. doi: 10.1006/jmbi.1996.0731
31. Rizza S, Filomeni G. Chronicles of a reductase: Biochemistry, genetics and physio-pathological role of GSNOR. *Free Radic Biol Med.* (2017) 110:19–30. doi: 10.1016/j.freeradbiomed.2017.05.014
32. Papaleo E. Integrating atomistic molecular dynamics simulations, experiments, and network analysis to study protein dynamics: strength in unity. *Front Mol Biosci.* (2015) 2:28. doi: 10.3389/fmolb.2015.00028

33. Papaleo E, Saladino G, Lambrughi M, Lindorff-Larsen K, Gervasio FL, Nussinov R. The role of protein loops and linkers in conformational dynamics and allostery. *Chem Rev.* (2016) 116:6391–423. doi: 10.1021/acs.chemrev.5b00623
34. Lambrughi M, De Gioia L, Gervasio FL, Lindorff-Larsen K, Nussinov R, Urani C, et al. DNA-binding protects p53 from interactions with cofactors involved in transcription-independent functions. *Nucleic Acids Res.* (2016) 44:9096–109. doi: 10.1093/nar/gkw770
35. Valimberti I, Tiberti M, Lambrughi M, Sarcevic B, Papaleo E. E2 superfamily of ubiquitin-conjugating enzymes: constitutively active or activated through phosphorylation in the catalytic cleft. *Sci Rep.* (2015) 5:14849. doi: 10.1038/srep14849
36. Tang C-H, Wei W, Liu L. Regulation of DNA repair by S-nitrosylation. *Biochim Biophys Acta* (2012) 1820:730–5. doi: 10.1016/j.bbagen.2011.04.014
37. Melino G, Bernassola F, Knight RA, Corasaniti MT, Nistico G, Finazzi-Agro A. S-nitrosylation regulates apoptosis. *Nature* (1997) 388:432–3. doi: 10.1038/41237
38. Rizza S, Filomeni G. Tumor suppressor roles of the denitrosylase GSNOR. *Crit Rev Oncog.* (2016) 21:433–45. doi: 10.1615/CritRevOncog.2017021074
39. Wei W, Li B, Hanes MA, Kakar S, Chen X, Liu L. S-nitrosylation from GSNOR deficiency impairs DNA repair and promotes hepatocarcinogenesis. *Sci Transl Med.* (2010) 2:19ra13. doi: 10.1126/scitranslmed.3000328
40. Rizza S, Montagna C, Cardaci S, Maiani E, Di G, Sanchez-quiles V, et al. S -nitrosylation of the mitochondrial chaperone TRAP1 sensitizes hepatocellular carcinoma cells to inhibitors of succinate Dehydrogenase. *Cancer Res.* (2016) 76:4170–82. doi: 10.1158/0008-5472.CAN-15-2637
41. Sciacovelli M, Guzzo G, Morello V, Frezza C, Zheng L, Nannini N, et al. The mitochondrial chaperone TRAP1 promotes neoplastic growth by inhibiting succinate dehydrogenase. *Cell Metab.* (2013) 17:988–99. doi: 10.1016/j.cmet.2013.04.019
42. Barnett SD, Buxton ILO. The role of S-nitrosoglutathione reductase (GSNOR) in human disease and therapy. *Crit Rev Biochem Mol Biol.* (2017) 52:340–54. doi: 10.1080/10409238.2017.1304353
43. Cañas A, López-Sánchez LM, Peñarando J, Valverde A, Conde F, Hernández V, et al. Altered S-nitrosothiol homeostasis provides a survival advantage to breast cancer cells in HER2 tumors and reduces their sensitivity to trastuzumab. *Biochim Biophys Acta* (2016) 1862:601–10. doi: 10.1016/j.bbadis.2016.02.005
44. Stamler JS, Simon DI, Osborne JA, Mullins ME, Jaraki O, Michel T, et al. S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. *Proc Natl Acad Sci USA.* (1992) 89:444–8.
45. Ambs S, Glynn SA. Candidate pathways linking inducible nitric oxide synthase to a basal-like transcription pattern and tumor progression in human breast cancer. *Cell Cycle* (2011) 10:619–24. doi: 10.4161/cc.10.4.14864
46. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov.* (2008) 7:156–67. doi: 10.1038/nrd2466
47. Rahman MA, Senga T, Ito S, Hyodo T, Hasegawa H, Hamaguchi M. S-nitrosylation at cysteine 498 of c-Src tyrosine kinase regulates nitric oxide-mediated cell invasion. *J Biol Chem.* (2010) 285:3806–14. doi: 10.1074/jbc.M109.059782
48. Switzer CH, Glynn SA, Cheng RY-S, Ridnour LA, Green JE, Ambs S, et al. S-nitrosylation of EGFR and Src activates an oncogenic signaling network in human basal-like breast cancer. *Mol Cancer Res.* (2012) 10:1203–15. doi: 10.1158/1541-7786.MCR-12-0124
49. Glynn SA, Boersma BJ, Dorsey TH, Yi M, Yfantis HG, Ridnour LA, et al. Increased NOS2 predicts poor survival in estrogen receptor-negative breast cancer patients. *J Clin Invest.* (2010) 120:3843–54. doi: 10.1172/JCI42059
50. Garrido P, Shalaby A, Walsh EM, Keane N, Webber M, Keane MM, et al. Impact of inducible nitric oxide synthase (iNOS) expression on triple negative breast cancer outcome and activation of EGFR and ERK signaling pathways. *Oncotarget* (2017) 8:80568–88. doi: 10.18632/oncotarget.19631
51. Kohno M, Pouyssegur J. Targeting the ERK signaling pathway in cancer therapy. *Ann Med.* (2006) 38:200–11. doi: 10.1080/07853890600551037
52. Lehmann BDB, Bauer J, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest.* (2011) 121:2750–767. doi: 10.1172/JCI45014DS1
53. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol.* (2008) 9:47–59. doi: 10.1038/nrm2308
54. Feng X, Sun T, Bei Y, Ding S, Zheng W, Lu Y, et al. S-nitrosylation of ERK inhibits ERK phosphorylation and induces apoptosis. *Sci Rep.* (2013) 3:1814. doi: 10.1038/srep01814
55. Gupta A, Anjomani-Virmouni S, Koundouros N, Dimitriadi M, Choo-Wing R, Valle A, et al. PARK2 depletion connects energy and oxidative stress to PI3K/Akt activation via PTEN S-Nitrosylation. *Mol Cell* (2017) 65:999–1013.e7. doi: 10.1016/j.molcel.2017.02.019
56. Kwak Y-D, Ma T, Diao S, Zhang X, Chen Y, Hsu J, et al. NO signaling and S-nitrosylation regulate PTEN inhibition in neurodegeneration. *Mol Neurodegener.* (2010) 5:49. doi: 10.1186/1750-1326-5-49
57. Martínez-Ruiz A, Villanueva L, González de Orduña C, López-Ferrer D, Higueras MA, Tarín C, et al. S-nitrosylation of Hsp90 promotes the inhibition of its ATPase and endothelial nitric oxide synthase regulatory activities. *Proc Natl Acad Sci USA.* (2005) 102:8525–30. doi: 10.1073/pnas.0407294102
58. Retzlaff M, Stahl M, Eberl HC, Lagleder S, Beck J, Kessler H, et al. Hsp90 is regulated by a switch point in the C-terminal domain. *EMBO Rep.* (2009) 10:1147–53. doi: 10.1038/embor.2009.153
59. Vigil D, Cherfils J, Rossman KL, Der CJ. Ras superfamily GEFs and GAPs: validated and tractable targets for cancer therapy? *Nat Rev Cancer* (2010) 10:842–57. doi: 10.1038/nrc2960
60. Karnoub AE, Weinberg RA. Ras oncogenes: split personalities. *Nat Rev Mol Cell Biol.* (2008) 9:517–31. doi: 10.1038/nrm2438
61. Raines KW, Cao G-L, Lee EK, Rosen GM, Shapiro P. Neuronal nitric oxide synthase-induced S-nitrosylation of H-Ras inhibits calcium ionophore-mediated extracellular-signal-regulated kinase activity. *Biochem J.* (2006) 397:329–36. doi: 10.1042/BJ20052002
62. Peretti M, Angelini M, Savalli N, Florio T, Yuspa SH, Mazzanti M. Chloride channels in cancer: Focus on chloride intracellular channel 1 and 4 (CLIC1 AND CLIC4) proteins in tumor development and as novel therapeutic targets. *Biochim Biophys Acta* (2015) 1848:2523–31. doi: 10.1016/j.bbamem.2014.12.012
63. Malik M, Jividen K, Padmakumar VC, Cataisson C, Li L, Lee J, et al. Inducible NOS-induced chloride intracellular channel 4 (CLIC4) nuclear translocation regulates macrophage deactivation. *Proc Natl Acad Sci USA.* (2012) 109:6130–5. doi: 10.1073/pnas.1201351109
64. Littler DR, Assaad NN, Harrop SJ, Brown LJ, Pankhurst GJ, Luciani P, et al. Crystal structure of the soluble form of the redox-regulated chloride ion channel protein CLIC4. *FEBS J.* (2005) 272:4996–5007. doi: 10.1111/j.1742-4658.2005.04909.x
65. Malik M, Shukla A, Amin P, Niedelman W, Lee J, Jividen K, et al. S-nitrosylation regulates nuclear translocation of chloride intracellular channel protein CLIC4. *J Biol Chem.* (2010) 285:23818–28. doi: 10.1074/jbc.M109.091611
66. Suh KS, Crutchley JM, Koochek A, Ryscavage A, Bhat K, Tanaka T, et al. Reciprocal modifications of CLIC4 in tumor epithelium and stroma mark malignant progression of multiple human cancers. *Clin Cancer Res.* (2007) 13:121–31. doi: 10.1158/1078-0432.CCR-06-1562
67. Peter ME, Hadji A, Murmann AE, Brockway S, Putzbach W, Pattanayak A, et al. The role of CD95 and CD95 ligand in cancer. *Cell Death Differ.* (2015) 22:549–59. doi: 10.1038/cdd.2015.3
68. Ivanov VN, Ronai Z, Hei TK. Opposite roles of FAP-1 and dynamin in the regulation of Fas (CD95) translocation to the cell surface and susceptibility to Fas ligand-mediated apoptosis. *J Biol Chem.* (2006) 281:1840–52. doi: 10.1074/jbc.M509866200
69. Igney FH, Krammer PH. Death and anti-death: tumour resistance to apoptosis. *Nat Rev Cancer* (2002) 2:277–88. doi: 10.1038/nrc776
70. Leon-Bollotte L, Subramaniam S, Cauvard O, Plenchette-Colas S, Paul C, Godard C, et al. S-nitrosylation of the death receptor fas promotes fas ligand-mediated apoptosis in cancer cells. *Gastroenterology* (2011) 140:2009–18. doi: 10.1053/j.gastro.2011.02.053
71. Gajate C, Mollinedo F. The antitumor ether lipid ET-18-OCH(3) induces apoptosis through translocation and capping of Fas/CD95 into membrane rafts in human leukemic cells. *Blood* (2001) 98:3860–3. doi: 10.1182/blood.V98.13.3860

72. Switzer CH, Cheng RY-S, Ridnour LA, Glynn SA, Ambs S, Wink DA. Ets-1 is a transcriptional mediator of oncogenic nitric oxide signaling in estrogen receptor-negative breast cancer. *Breast Cancer Res.* (2012) 14:R125. doi: 10.1186/bcr3319
73. Dittmer J. The biology of the Ets1 proto-oncogene. *Mol Cancer* (2003) 2:29. doi: 10.1186/1476-4598-2-29
74. Lincoln DW, Bove K. The transcription factor Ets-1 in breast cancer. *Front Biosci.* (2005) 10:506–11. doi: 10.2741/1546
75. Charafe-Jauffret E, Ginestier C, Monville F, Finetti P, Adélaïde J, Cervera N, et al. Gene expression profiling of breast cell lines identifies potential new basal markers. *Oncogene* (2006) 25:2273–84. doi: 10.1038/sj.onc.1209254
76. Withana NP, Blum G, Sameni M, Slaney C, Anbalagan A, Olive MB, et al. Cathepsin B inhibition limits bone metastasis in breast cancer. *Cancer Res.* (2012) 72:1199–209. doi: 10.1158/0008-5472.CAN-11-2759
77. Sánchez-Muñoz A, Gallego E, de Luque V, Pérez-Rivas LG, Vicioso L, Ribelles N, et al. Lack of evidence for KRAS oncogenic mutations in triple-negative breast cancer. *BMC Cancer* (2010) 10:136. doi: 10.1186/1471-2407-10-136
78. Chatterjee M, Ben-Josef E, Thomas DG, Morgan MA, Zalupski MM, Khan G, et al. Caveolin-1 is associated with tumor progression and confers a multi-modality resistance phenotype in pancreatic cancer. *Sci Rep.* (2015) 5:10867. doi: 10.1038/srep10867
79. Thompson TC, Tahir SA, Li L, Watanabe M, Naruishi K, Yang G, et al. The role of caveolin-1 in prostate cancer: clinical implications. *Prostate Cancer Prostatic Dis.* (2010) 13:6–11. doi: 10.1038/pcan.2009.29
80. Ho C-C, Huang P-H, Huang H-Y, Chen Y-H, Yang P-C, Hsu S-M. Up-regulated caveolin-1 accentuates the metastasis capability of lung adenocarcinoma by inducing filopodia formation. *Am J Pathol.* (2002) 161:1647–56. doi: 10.1016/S0002-9440(10)64442-2
81. Chanvorachote P, Nimmannit U, Lu Y, Talbott S, Jiang B-H, Rojanasakul Y. Nitric oxide regulates lung carcinoma cell anoikis through inhibition of ubiquitin-proteasomal degradation of caveolin-1. *J Biol Chem.* (2009) 284:28476–84. doi: 10.1074/jbc.M109.050864
82. Muz B, de la Puente P, Azab F, Azab AK. The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. *Hypoxia* (2015) 3:83–92. doi: 10.2147/HP.S93413
83. Vaupel P. The role of hypoxia-induced factors in tumor progression. *Oncologist* (2004) 9(Suppl. 5):10–7. doi: 10.1634/theoncologist.9-90005-10
84. Salceda S, Caro J. Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J Biol Chem.* (1997) 272:22642–7.
85. Kallio PJ, Pongratz I, Gradin K, McGuire J, Poellinger L. Activation of hypoxia-inducible factor 1alpha: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor. *Proc Natl Acad Sci USA.* (1997) 94:5667–72.
86. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, et al. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science* (2001) 292:464–8. doi: 10.1126/science.1059817
87. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, et al. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* (1999) 399:271–5. doi: 10.1038/20459
88. Li F, Sonveaux P, Rabbani ZN, Liu S, Yan B, Huang Q, et al. Regulation of HIF-1alpha stability through S-nitrosylation. *Mol Cell* (2007) 26:63–74. doi: 10.1016/j.molcel.2007.02.024
89. Moeller BJ, Cao Y, Li CY, Dewhirst MW. Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: role of reoxygenation, free radicals, and stress granules. *Cancer Cell* (2004) 5:429–41. doi: 10.1016/S1535-6108(04)00115-1
90. Cramer T, Johnson RS. A novel role for the hypoxia inducible transcription factor HIF-1alpha: critical regulation of inflammatory cell function. *Cell Cycle* (2003) 2:192–3.
91. Johnson JL. Evolution and function of diverse Hsp90 homologs and cochaperone proteins. *Biochim Biophys Acta* (2012) 1823:607–13. doi: 10.1016/j.bbamcr.2011.09.020
92. Papaleo E, Parravicini F, Grandori R, De Gioia L, Brocca S. Structural investigation of the cold-adapted acylaminoacyl peptidase from Sporosarcina psychrophila by atomistic simulations and biophysical methods. *Biochim Biophys Acta* (2014) 1844:2203–13. doi: 10.1016/j.bbapap.2014.09.018
93. Nygaard M, Terkelsen T, Olsen AV, Sora V, Salamanca J, Rizza F, et al. The mutational landscape of the oncogenic MZF1 SCAN domain in cancer. *Front Mol Biosci.* (2016) 3:78. doi: 10.3389/fmolb.2016.00078
94. Mathiassen SG, Larsen IB, Poulsen EG, Madsen CT, Papaleo E, Lindorff-Larsen K, et al. A two-step protein quality control pathway for a misfolded DJ-1 variant in fission yeast. *J Biol Chem.* (2015) 290:21141–53. doi: 10.1074/jbc.M115.662312
95. Nielsen SV, Stein A, Dinitzen AB, Papaleo E, Tatham MH, Poulsen EG, et al. Predicting the impact of Lynch syndrome-causing missense mutations from structural calculations. *PLoS Genet.* (2017) 13:e1006739. doi: 10.1371/journal.pgen.1006739
96. Desgrosellier JS, Cheresh DA. Integrins in cancer: biological implications and therapeutic opportunities. *Nat Rev Cancer* (2010) 10:9–22. doi: 10.1038/nrc2748
97. Isaac J, Tarapore P, Zhang X, Lam Y-W, Ho S-M. Site-specific S-nitrosylation of integrin α6 increases the extent of prostate cancer cell migration by enhancing integrin β1 association and weakening adherence to laminin-1. *Biochemistry* (2012) 51:9689–97. doi: 10.1021/bi3012324
98. Aaltoma SH, Lipponen PK, Kosma VM. Inducible nitric oxide synthase (iNOS) expression and its prognostic value in prostate cancer. *Anticancer Res.* (2001) 21:3101–6. 101.
99. Yuan K, Liu Y, Chen H-N, Zhang L, Lan J, Gao W, et al. Thiol-based redox proteomics in cancer research. *Proteomics* (2015) 15:287–299. doi: 10.1002/pmic.201400164
100. Paige JS, Xu G, Stancevic B, Jaffrey SR. Nitrosothiol reactivity profiling identifies S-nitrosylated proteins with unexpected stability. *Chem Biol.* (2008) 15:1307–16. doi: 10.1016/j.chembiol.2008.10.013
101. Torta F, Usuelli V, Malgaroli A, Bachi A. Proteomic analysis of protein S-nitrosylation. *Proteomics* (2008) 8:4484–94. doi: 10.1002/pmic.200800089
102. Murray CI, Uhrigshardt H, O'Meally RN, Cole RN, Van Eyk JE. Identification and Quantification of S-Nitrosylation by Cysteine Reactive Tandem Mass Tag Switch Assay. *Mol Cell Proteomics* (2012) 11:M111.013441. doi: 10.1074/mcp.M111.013441
103. Yang J, Carroll KS, Liebler DC. The expanding landscape of the thiol redox proteome. *Mol Cell Proteomics* (2016) 15:1–11. doi: 10.1074/mcp.O115.056051
104. Lee TY, Chen YJ, Lu CT, Ching WC, Teng YC, Huang H, et al. dbSNO: A database of cysteine S-nitrosylation. *Bioinformatics* (2012) 28:2293–5. doi: 10.1093/bioinformatics/bts436
105. Chen Y-J, Lu C-T, Su M-G, Huang K-Y, Ching W-C, Yang H-H, et al. dbSNO 2.0: a resource for exploring structural environment, functional and disease association and regulatory network of protein S-nitrosylation. *Nucleic Acids Res.* (2014) 43:D503–11. doi: 10.1093/nar/gku1176
106. Olsson MHM, Søndergaard CR, Rostkowski M, Jensen JH. PROPKA3: Consistent treatment of internal and surface residues in empirical pKa Predictions. *J Chem Theory Comput.* (2011) 7:525–37. doi: 10.1021/ct100578z
107. Marino SM. Protein flexibility and cysteine reactivity: influence of mobility on the H-bond network and effects on pKa prediction. *Protein J.* (2014) 33:323–36. doi: 10.1007/s10930-014-9564-z
108. Marino SM, Gladyshev VN. Structural analysis of cysteine S-Nitrosylation: a modified acid-based motif and the emerging role of trans-nitrosylation. *J Mol Biol.* (2010) 395:844–59. doi: 10.1016/j.jmb.2009.10.042
109. Xue Y, Liu Z, Gao X, Jin C, Wen L, Yao X, et al. GPS-SNO: Computational prediction of protein s-nitrosylation sites with a modified GPS algorithm. *PLoS ONE* (2010) 5:e0011290. doi: 10.1371/journal.pone.0011290
110. Li B-Q, Hu L-L, Niu S, Cai Y-D, Chou K-C. Predict and analyze S-nitrosylation modification sites with the mRMR and IFS approaches. *J Proteomics* (2012) 75:1654–65. doi: 10.1016/j.jprot.2011.12.003
111. Hao G, Derakhshan B, Shi L, Campagne F, Gross SS. SNOSID, a proteomic method for identification of cysteine S-nitrosylation sites in complex protein mixtures. *Proc Natl Acad Sci USA* (2006) 103:1012–7. doi: 10.1073/pnas.0508412103

112. Lee TY, Chen YJ, Lu TC, Huang H, Chen YJ. Snosite: Exploiting maximal dependence decomposition to identify cysteine S-Nitrosylation with substrate site specificity. *PLoS ONE* (2011) 6:e0021849. doi: 10.1371/journal.pone.0021849
113. Li Y-X, Shao Y-H, Jing L, Deng N-Y. An efficient support vector machine approach for identifying protein S-nitrosylation sites. *Protein Pept Lett.* (2011) 18:573–87. doi: 10.2174/092986611795222731
114. Xu Y, Ding J, Wu LY, Chou KC. iSNO-PseAAC: predict cysteine s-nitrosylation sites in proteins by incorporating position specific amino acid propensity into pseudo amino acid composition. *PLoS ONE* (2013) 8:e0055844. doi: 10.1371/journal.pone.0055844
115. Xu Y, Shao X, Wu L, Deng N, Chou K. iSNO-AAPair: incorporating amino acid pairwise coupling into PseAAC for predicting cysteine S-nitrosylation sites in proteins. *PeerJ* (2013) 1:e171. doi: 10.7717/peerj.171
116. Zhang J, Zhao X, Sun P, Ma Z. PSNO: predicting cysteine S-nitrosylation sites by incorporating various sequence-derived features into the general form of Chou's PseAAC. *Int J Mol Sci.* (2014) 15:11204–19. doi: 10.3390/ijms150711204
117. Huang G, Lu L, Feng K, Zhao J, Zhang Y, Xu Y, et al. Prediction of S-nitrosylation modification sites based on kernel sparse representation classification and mRMR algorithm. *Biomed Res Int.* (2014) 2014:438341. doi: 10.1155/2014/438341
118. Jia C, Lin X, Wang Z. Prediction of protein S-nitrosylation sites based on adapted normal distribution bi-profile Bayes and Chou's pseudo amino acid composition. *Int J Mol Sci.* (2014) 15:10410–23. doi: 10.3390/ijms150610410
119. Smith BC, Marletta MA. Mechanisms of S-nitrosothiol formation and selectivity in nitric oxide signaling. *Curr Opin Chem Biol.* (2012) 16:498–506. doi: 10.1016/j.cbpa.2012.10.016
120. Bhatnagar A, Bandyopadhyay D. Characterization of cysteine thiol modifications based on protein microenvironments and local secondary structures. *Proteins* (2017) 86:192–209. doi: 10.1002/prot.25424
121. Greco TM, Hodara R, Parastatidis I, Heijnen HFG, Dennehy MK, Liebler DC, et al. Identification of S-nitrosylation motifs by site-specific mapping of the S-nitrosocysteine proteome in human vascular smooth muscle cells. *Proc Natl Acad Sci USA.* (2006) 103:7420–5. doi: 10.1073/pnas.0600729103
122. Stamler JS, Toone EJ, Lipton SA, Sucher NJ. (S)NO signals: translocation, regulation, and a consensus motif. *Neuron* (1997) 18:691–6. doi: 10.1016/S0896-6273(00)80310-4
123. Stamler JS, Jia L, Eu JP, McMahon TJ, Demchenko IT, Bonaventura J, et al. Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient. *Science* (1997) 276:2034–7.
124. Lindermayr C, Saalbach G, Bahnweg G, Durner J. Differential inhibition of arabidopsis methionine adenosyltransferases by protein S-nitrosylation. *J Biol Chem.* (2006) 281:4285–91. doi: 10.1074/jbc.M511635200
125. Pérez-Mato I, Castro C, Ruiz FA, Corrales FJ, Mato JM. Methionine adenosyltransferase S-nitrosylation is regulated by the basic and acidic amino acids surrounding the target thiol. *J Biol Chem.* (1999) 274:17075–79. doi: 10.1074/jbc.274.24.17075
126. Kim SO, Merchant K, Nudelman R, Beyer WF, Keng T, DeAngelo J, et al. OxyR: A molecular code for redox-related signaling. *Cell* (2002) 109:383–96. doi: 10.1016/S0092-8674(02)00723-7
127. Doulias P-T, Greene JL, Greco TM, Tenopoulou M, Seeholzer SH, Dunbrack RL, et al. Structural profiling of endogenous S-nitrosocysteine residues reveals unique features that accommodate diverse mechanisms for protein S-nitrosylation. *Proc Natl Acad Sci USA.* (2010) 107:16958–63. doi: 10.1073/pnas.1008036107
128. Hess DT, Matsumoto A, Kim S-O, Marshall HE, Stamler JS. Protein S-nitrosylation: purview and parameters. *Nat Rev Mol Cell Biol.* (2005) 6:150–66. doi: 10.1038/nrm1569
129. Foster MW, Forrester MT, Stamler JS. A protein microarray-based analysis of S-nitrosylation. *Proc Natl Acad Sci USA.* (2009) 106:18948–53. doi: 10.1073/pnas.0900729106
130. Chen Y-J, Ku W-C, Lin P-Y, Chou H-C, Khoo K-H, Chen Y-J. S-alkylating labeling strategy for site-specific identification of the s-nitrosoproteome. *J Proteome Res.* (2010) 9:6417–39. doi: 10.1021/pr100680a
131. Kornberg MD, Sen N, Hara MR, Juluri KR, Nguyen JVK, Snowman AM, et al. GAPDH mediates nitrosylation of nuclear proteins. *Nat Cell Biol.* (2010) 12:1094–100. doi: 10.1038/ncb2114
132. Jia J, Arif A, Terenzi F, Willard B, Plow EF, Hazen SL, et al. Target-selective protein S-nitrosylation by sequence motif recognition. *Cell* (2014) 159:623–34. doi: 10.1016/j.cell.2014.09.032
133. Liu X, Miller MJ, Joshi MS, Thomas DD, Lancaster JR. Accelerated reaction of nitric oxide with O2 within the hydrophobic interior of biological membranes. *Proc Natl Acad Sci USA.* (1998) 95:2175–9.
134. Zhang H, Andrekopoulos C, Xu Y, Joseph J, Hogg N, Feix J, Kalyanaraman B. Decreased S-nitrosation of peptide thiols in the membrane interior. *Free Radic Biol Med.* (2009) 47:962–8. doi: 10.1016/j.freeradbiomed.2009.06.031
135. Nedospasov A, Rafikov R, Beda N, Nudler E. An autocatalytic mechanism of protein nitrosylation. *Proc Natl Acad Sci USA.* (2000) 97:13543–8. doi: 10.1073/pnas.250398197
136. Kim SF, Huri DA, Snyder SH. Inducible nitric oxide synthase binds, S-nitrosylates, and activates cyclooxygenase-2. *Science* (2005) 310:1966–70. doi: 10.1126/science.1119407
137. Eu JP, Sun J, Xu L, Stamler JS, Meissner G. The skeletal muscle calcium release channel: coupled O2 sensor and NO signaling functions. *Cell* (2000) 102:499–509. doi: 10.1016/S0092-8674(00)00054-4
138. Sun J, Xin C, Eu JP, Stamler JS, Meissner G. Cysteine-3635 is responsible for skeletal muscle ryanodine receptor modulation by NO. *Proc Natl Acad Sci USA.* (2001) 98:11158–62. doi: 10.1073/pnas.201289098
139. Bosworth CA, Toledo JC, Zmijewski JW, Li Q, Lancaster JR. Dinitrosyliron complexes and the mechanism(s) of cellular protein nitrosothiol formation from nitric oxide. *Proc Natl Acad Sci USA.* (2009) 106:4671–6. doi: 10.1073/pnas.0710416106
140. Foster MW, McMahon TJ, Stamler JS. S-nitrosylation in health and disease. *Trends Mol Med.* (2003) 9:160–8. doi: 10.1016/S1471-4914(03)00028-5
141. Fowler NJ, Blanford CF, De Visser SP, Warwicker J. Features of reactive cysteines discovered through computation: from kinase inhibition to enrichment around protein degrons. *Sci Rep.* (2017) 7:1–12. doi: 10.1038/s41598-017-15997-z
142. Straub AC, Billaud M, Johnstone SR, Best AK, Yemen S, Dwyer ST, et al. Compartmentalized connexin 43 s-nitrosylation/denitrosylation regulates heterocellular communication in the vessel wall. *Arterioscler Thromb Vasc Biol.* (2011) 31:399–407. doi: 10.1161/ATVBAHA.110.215939
143. Iwakiri Y. S-nitrosylation of proteins: a new insight into endothelial cell function regulated by eNOS-derived NO. *Nitric oxide Biol Chem.* (2011) 25:95–101. doi: 10.1016/j.niox.2011.04.014
144. Mazmanian K, Sargsyan K, Grauffel C, Dudev T, Lim C. preferred hydrogen-bonding partners of cysteine: implications for regulating Cys functions. *J Phys Chem B* (2016) 120:10288–96. doi: 10.1021/acs.jpcb.6b08109
145. Rosenfeld RJ, Bonaventura J, Szymczyna BR, MacCoss MJ, Arvai AS, Yates JR, et al. Nitric-oxide synthase forms N-NO-pterin and S-NO-cys: implications for activity, allostery, and regulation. *J Biol Chem.* (2010) 285:31581–9. doi: 10.1074/jbc.M109.072496
146. Choi YB, Tenneti L, Le DA, Ortiz J, Bai G, Chen HS, et al. Molecular basis of NMDA receptor-coupled ion channel modulation by S-nitrosylation. *Nat Neurosci.* (2000) 3:15–21. doi: 10.1038/71090
147. Broillet MC. A single intracellular cysteine residue is responsible for the activation of the olfactory cyclic nucleotide-gated channel by NO. *J Biol Chem.* (2000) 275:15135–41. doi: 10.1074/jbc.275.20.15135
148. Stuehr DJ. Mammalian nitric oxide synthases. *Biochim Biophys Acta* (1999) 1411:217–30.
149. Hemmens B, Goessler W, Schmidt K, Mayer B. Role of bound zinc in dimer stabilization but not enzyme activity of neuronal nitric-oxide synthase. *J Biol Chem.* (2000) 275:35786–91. doi: 10.1074/jbc.M005976200
150. Li H, Raman CS, Glaser CB, Blasko E, Young TA, Parkinson JF, et al. Crystal structures of zinc-free and -bound heme domain of human inducible nitric-oxide synthase. Implications for dimer stability and comparison with endothelial nitric-oxide synthase. *J Biol Chem.* (1999) 274:21276–84.
151. Nagpal L, Haque MM, Saha A, Mukherjee N, Ghosh A, Ranu BC, et al. Mechanism of inducible nitric-oxide synthase dimerization inhibition by novel pyrimidine imidazoles. *J Biol Chem.* (2013) 288:19685–97. doi: 10.1074/jbc.M112.446542
152. Cortese-Krott MM, Kulakov L, Opländer C, Kolb-Bachofen V, Kröncke K-D, Suschek C V. Zinc regulates iNOS-derived nitric oxide formation in endothelial cells. *Redox Biol* (2014) 2:945–54. doi: 10.1016/j.redox.2014.06.011

153. McMillan K, Adler M, Auld DS, Baldwin JJ, Blasko E, Browne LJ, et al. Allosteric inhibitors of inducible nitric oxide synthase dimerization discovered via combinatorial chemistry. *Proc Natl Acad Sci USA*. (2000) 97:1506–11. doi: 10.1073/pnas.97.4.1506
154. Osawa Y, Lowe ER, Everett AC, Dunbar AY, Billecke SS. Proteolytic degradation of nitric oxide synthase: effect of inhibitors and role of hsp90-based chaperones. *J Pharmacol Exp Ther*. (2003) 304:493–7. doi: 10.1124/jpet.102.035055
155. Zou M-H, Shi C, Cohen RA. Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J Clin Invest*. (2002) 109:817–26. doi: 10.1172/JCI14442
156. Chen Y, Panda K, Stuehr DJ. Control of nitric oxide synthase dimer assembly by a heme-NO-dependent mechanism. *Biochemistry* (2002) 41:4618–25. doi: 10.1021/bi011877t
157. Rabender CS, Alam A, Sundaresan G, Cardnell RJ, Yakovlev VA, Mukhopadhyay ND, et al. The role of nitric oxide synthase uncoupling in tumor progression. *Mol Cancer Res*. (2015) 13:1034–43. doi: 10.1158/1541-7786.MCR-15-0057-T
158. Ravi K, Brennan LA, Levic S, Ross PA, Black SM. S-nitrosylation of endothelial nitric oxide synthase is associated with monomerization and decreased enzyme activity. *Proc Natl Acad Sci USA*. (2004) 101:2619–24. doi: 10.1073/pnas.0300464101
159. Lipton SA, Choi YB, Pan ZH, Lei SZ, Chen HS, Sucher NJ, et al. A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* (1993) 364:626–32. doi: 10.1038/364626a0
160. Lipton SA, Choi Y-B, Takahashi H, Zhang D, Li W, Godzik A. Cysteine regulation of protein function–as exemplified by NMDA-receptor modulation. *Trends Neurosci* (2002) 25:474–80. doi: 10.1016/S0166-2236(02)02245-2
161. Kumar V, Martin F, Hahn MG, Schaefer M, Stamler JS, Stasch J-P, et al. Insights into BAY 60-2770 activation and S-nitrosylation-dependent desensitization of soluble guanylyl cyclase via crystal structures of homologous nostoc H-NOX domain complexes. *Biochemistry* (2013) 52:3601–8. doi: 10.1021/bi301657w
162. Chan NL, Rogers PH, Arnone A. Crystal structure of the S-nitroso form of liganded human hemoglobin. *Biochemistry* (1998) 37:16459–64. doi: 10.1021/bi9816711
163. Eichmann C, Tzitzilonis C, Nakamura T, Kwiatkowski W, Maslennikov I, Choe S, et al. S-Nitrosylation Induces Structural and Dynamical Changes in a Rhodanese Family Protein. *J Mol Biol*. (2016) 428:3737–51. doi: 10.1016/j.jmb.2016.07.010
164. Živković ML, Zaręba-Kozioł M, Zhukova L, Poznanski J, Zhukov I, Wysłouch-Cieszynska A. Post-translational S-nitrosylation is an endogenous factor fine tuning the properties of human S100A1 protein. *J Biol Chem*. (2012) 287:40457–70. doi: 10.1074/jbc.M112.418392
165. Tsai CJ, del Sol A, Nussinov R. Allostery: absence of a change in shape does not imply that allostery is not at play. *J Mol Biol*. (2008) 378:1–11. doi: 10.1016/j.jmb.2008.02.034
166. Collier G, Ortiz V. Emerging computational approaches for the study of protein allostery. *Arch Biochem Biophys*. (2013) 538:6–15. doi: 10.1016/j.abb.2013.07.025
167. Hertig S, Latorraca NR, Dror RO. Revealing atomic-level mechanisms of protein allostery with molecular dynamics simulations. *PLoS Comput Biol*. (2016) 12:e1004746. doi: 10.1371/journal.pcbi.1004746
168. Feher VA, Durrant JD, Van Wart AT, Amaro RE. Computational approaches to mapping allosteric pathways. *Curr Opin Struct Biol*. (2014) 25:98–103. doi: 10.1016/j.sbi.2014.02.004
169. Fleuren EDG, Zhang L, Wu J, Daly RJ. The kinome "at large" in cancer. *Nat Rev Cancer* (2016) 16:83–98. doi: 10.1038/nrc.2015.18
170. Olow A, Chen Z, Niedner RH, Wolf DM, Yau C, Pankov A, et al. An atlas of the human kinome reveals the mutational landscape underlying dysregulated phosphorylation cascades in cancer. *Cancer Res*. (2016) 76:1733–45. doi: 10.1158/0008-5472.CAN-15-2325-T
171. Fu W, Hall JE, Schaller MD, Babb M, Cancer R, Virginia W. Focal adhesion kinase-regulated signaling events in human cancer. *BioMol Concepts* (2012) 3:225–40. doi: 10.1515/bmc-2011-0049
172. Corcoran A, Cotter TG. Redox regulation of protein kinases. *FEBS J*. (2013) 280:1944–65. doi: 10.1111/febs.12224
173. Andre FR, dos Santos PF, Rando DG. Theoretical studies of the role of C-terminal cysteines in the process of S-nitrosylation of human Src kinases. *J Mol Model*. (2016) 22:1–10. doi: 10.1007/s00894-015-2892-x
174. Mueller RP, Huber JR. Two rotational isomers of methyl thionitrite: light-induced, reversible isomerization in an argon matrix. *J Phys Chem*. (1984) 88:1605–8. doi: 10.1021/j150652a033
175. Cánneva A, Della Védova CO, Mitzel NW, Erben MF. Conformational properties of ethyl- and 2,2,2-trifluoroethyl thionitrites, (CX3CH2SNO, X = H and F). *J Phys Chem A* (2015) 119:1524–33. doi: 10.1021/jp507406w
176. Canneva A, Erben MF, Romano RM, Vishnevskiy Y V, Reuter CG, Mitzel NW, et al. The structure and Conformation of (CH3)3 CSNO. *Chemistry* (2015) 21:10436–42. doi: 10.1002/chem.201500811
177. Miljkovic JL, Kenkel I, Ivanović-Burmazović I, Filipovic MR. Generation of HNO and HSNO from nitrite by heme-iron-catalyzed metabolism with H2S. *Angew Chem Int Ed Engl*. (2013) 52:12061–4. doi: 10.1002/anie.201305669
178. Filipovic MR, Miljkovic JL, Nauser T, Royzen M, Klos K, Shubina T, et al. Chemical characterization of the smallest S-nitrosothiol, HSNO; cellular cross-talk of H2S and S-nitrosothiols. *J Am Chem Soc*. (2012) 134:12016–27. doi: 10.1021/ja3009693
179. Ivanova LV, Anton BJ, Timerghazin QK. On the possible biological relevance of HSNO isomers: a computational investigation. *Phys Chem Chem Phys*. (2014) 16:8476–86. doi: 10.1039/C4CP00469H
180. Nava M, Martin-Drumel M-A, Lopez CA, Crabtree KN, Womack CC, Nguyen TL, et al. Spontaneous and selective formation of HSNO, a crucial intermediate linking H2S and nitroso chemistries. *J Am Chem Soc*. (2016) 138:11441–4. doi: 10.1021/jacs.6b05886
181. Timerghazin QK, English AM, Peslherbe GH. On the multireference character of S-nitrosothiols: a theoretical study of HSNO. *Chem Phys Lett*. (2008) 454:24–9. doi: 10.1016/j.cplett.2008.01.062
182. Baciu C, Cho K-B, Gauld JW. Influence of Cu+ on the RS-NO bond dissociation energy of S-nitrosothiols. *J Phys Chem B* (2005) 109:1334–6. doi: 10.1021/jp0443759
183. Méndez M, Francisco JS, Dixon DA. Thermodynamic properties of the isomers of [HNOS], [HNO2 S], and [HNOS2] and the role of the central sulfur. *Chemistry* (2014) 20:10231–5. doi: 10.1002/chem.201404076
184. Timerghazin QK, Peslherbe GH, English AM. Structure and stability of HSNO, the simplest S-nitrosothiol. *Phys Chem Chem Phys*. (2008) 10:1532–9. doi: 10.1039/b715025c
185. Timerghazin QK, Talipov MR. Unprecedented external electric field effects on *S* -nitrosothiols: possible mechanism of biological regulation? *J Phys Chem Lett*. (2013) 4:1034–8. doi: 10.1021/jz400354m
186. Khomyakov DG, Timerghazin QK. Toward reliable modeling of S-nitrosothiol chemistry: Structure and properties of methyl thionitrite (CH_3SNO), an S-nitrosocysteine model. *J Chem Phys*. (2017) 147:44305. doi: 10.1063/1.4995300
187. Meyer B, Genoni A, Boudier A, Leroy P, Ruiz-Lopez MF. Structure and stability studies of pharmacologically relevant *S* -Nitrosothiols: a theoretical approach. *J Phys Chem A* (2016) 120:4191–200. doi: 10.1021/acs.jpca.6b02230
188. Talipov MR, Timerghazin QK. Protein Control of *S* -nitrosothiol reactivity: interplay of antagonistic resonance structures. *J Phys Chem B* (2013) 117:1827–37. doi: 10.1021/jp310664z
189. Marazzi M, López-Delgado A, Fernández-González MA, Castaño O, Frutos LM, Temprado M. Modulating nitric oxide release by S-nitrosothiol photocleavage: mechanism and substituent effects. *J Phys Chem A* (2012) 116:7039–49. doi: 10.1021/jp304707n
190. Nagy B, Szakács P, Csontos J, Rolik Z, Tasi G, Kállay M. High-accuracy theoretical thermochemistry of atmospherically important sulfur-containing molecules. *J Phys Chem A* (2011) 115:7823–33. doi: 10.1021/jp203406d
191. Zhou C, Liang J, Cheng S, Shi T, Houk KN, Wei D-Q, et al. *Ab initio* molecular metadynamics simulation for S-nitrosylation by nitric oxide: S-nitroxide as the key intermediate. *Mol Simul*. (2017) 43:1134–41. doi: 10.1080/08927022.2017.1319059
192. Timerghazin QK, Peslherbe GH, English AM. Resonance description of S-nitrosothiols: Insights into reactivity. *Org Lett*. (2007) 9:3049–52. doi: 10.1021/ol0711016

193. Baciu C, Gauld JW. An assessment of theoretical methods for the calculation of accurate structures and SN bond dissociation energies of *S* -Nitrosothiols (RSNOs). *J Phys Chem A* (2003) 107:9946–52. doi: 10.1021/jp035205j
194. Ivanova LV, Cibich D, Deye G, Talipov MR, Timerghazin QK. Modeling of S-Nitrosothiol-Thiol reactions of biological significance: HNO production by S-Thiolation requires a proton shuttle and stabilization of polar intermediates. *Chembiochem* (2017) 18:726–38. doi: 10.1002/cbic.201600556
195. Gao Y, Dai Y, Wu G. Solid-State 15N and 17O NMR Studies of S-Nitrosothiols. *J Phys Chem B* (2017) 121:7311–7. doi: 10.1021/acs.jpcb.7b05685
196. Wang K, Hou Y, Zhang W, Ksebati MB, Xian M, Cheng JP, et al. 15N NMR and electronic properties of S-nitrosothiols. *Bioorg Med Chem Lett.* (1999) 9:2897–902.
197. Hou Y, Wang J, Arias F, Echegoyen L, Wang PG. Electrochemical studies of S-nitrosothiols. *Bioorg Med Chem Lett.* (1998) 8:3065–70. doi: 10.1016/S0960-894X(98)00563-0
198. Gaucher C, Boudier A, Dahboul F, Parent M, Leroy P. S-nitrosation/denitrosation in cardiovascular pathologies: facts and concepts for the rational design of S-nitrosothiols. *Curr Pharm Des.* (2013) 19:458–72. doi: 10.2174/138161213804143635
199. Koppenol WH. Nitrosation, thiols, and hemoglobin: energetics and kinetics. *Inorg Chem* (2012) 51:5637–41. doi: 10.1021/ic202561f
200. Toubin C, Yeung DY-H, English AM, Peslherbe GH. Theoretical evidence that Cu(I) complexation promotes degradation of S-nitrosothiols. *J Am Chem Soc.* (2002) 124:14816–7. doi: 10.1021/ja027386t
201. Ivanova LV, Anton BJ, Timerghazin QK. On the possible biological relevance of HSNO isomers: a computational investigation. *Phys Chem Chem Phys.* (2014) 16:8476–86. doi: 10.1039/c4cp00469h
202. Arulsamy N, Bohle DS, Butt JA, Irvine GJ, Jordan A, Sagan E. Interrelationships between conformational dynamics and the redox chemistry of S-Nitrosothiols. *J Am Chem Soc.* (1999) 121:7115–23. doi: 10.1021/JA9901314
203. Bartberger MD, Houk KN, Powell SC, Mannion JD, Lo KY, Stamler JS, et al. Theory, Spectroscopy, and crystallographic analysis of *s* -nitrosothiols: conformational distribution dictates spectroscopic behavior. *J Am Chem Soc.* (2000) 122:5889–90. doi: 10.1021/ja994476y
204. Roy B, du Moulinet d'Hardemare A, Fontecave M. New thionitrites: synthesis, stability, and nitric oxide generation. *J Org Chem.* (1994) 59:7019–26. doi: 10.1021/jo00102a028
205. Wang PG, Xian M, Tang X, Wu X, Wen Z, Cai T, et al. Nitric oxide donors: chemical activities and biological applications. *Chem Rev.* (2002) 102:1091–134. doi: 10.1021/cr000040l
206. Han S. Force field parameters for S-nitrosocysteine and molecular dynamics simulations of S-nitrosated thioredoxin. *Biochem Biophys Res Commun.* (2008) 377:612–6. doi: 10.1016/j.bbrc.2008.10.017
207. Weichsel A, Brailey JL, Montfort WR. Buried S-nitrosocysteine revealed in crystal structures of human thioredoxin. *Biochemistry* (2007) 46:1219–27. doi: 10.1021/bi061878r
208. Petrov D, Margreitter C, Grandits M, Oostenbrink C, Zagrovic B. A systematic framework for molecular dynamics simulations of protein post-translational modifications. *PLoS Comput Biol.* (2013) 9:e1003154. doi: 10.1371/journal.pcbi.1003154
209. Margreitter C, Petrov D, Zagrovic B. Vienna-PTM web server: a toolkit for MD simulations of protein post-translational modifications. *Nucleic Acids Res.* (2013) 41:W422–6. doi: 10.1093/nar/gkt416
210. Koziara KB, Stroet M, Malde AK, Mark AE. Testing and validation of the automated topology builder (ATB) version 2.0: prediction of hydration free enthalpies. *J Comput Aided Mol Des.* (2014) 28:221–33. doi: 10.1007/s10822-014-9713-7
211. Malde AK, Zuo L, Breeze M, Stroet M, Poger D, Nair PC,et al. An automated force field topology builder (ATB) and repository: Version 1.0. *J Chem Theory Comput.* (2011) 7:4026–37. doi: 10.1021/ct200196m
212. Chen YY, Chu HM, Pan KT, Teng CH, Wang DL, Wang AHJ, et al. Cysteine S-nitrosylation protects protein-tyrosine phosphatase 1B against oxidation-induced permanent inactivation. *J Biol Chem.* (2008) 283:35265–72. doi: 10.1074/jbc.M805287200
213. Choi MS, Nakamura T, Cho S-J, Han X, Holland EA, Qu J, et al. Transnitrosylation from DJ-1 to PTEN attenuates neuronal cell death in parkinson's disease models. *J Neurosci.* (2014) 34:15123–31. doi: 10.1523/JNEUROSCI.4751-13.2014
214. Schreiter ER, Rodríguez MM, Weichsel A, Montfort WR, Bonaventura J. S-nitrosylation-induced conformational change in blackfin tuna myoglobin. *J Biol Chem.* (2007) 282:19773–80. doi: 10.1074/jbc.M701363200
215. Chowdhury R, Flashman E, Mecinović J, Kramer HB, Kessler BM, Frapart YM, et al. Studies on the reaction of nitric oxide with the hypoxia-inducible factor prolyl hydroxylase domain 2 (EGLN1). *J Mol Biol.* (2011) 410:268–79. doi: 10.1016/j.jmb.2011.04.075
216. Skaff DA, Ramyar KX, McWhorter WJ, Barta ML, Geisbrecht BV, Miziorko HM. Biochemical and structural basis for inhibition of *Enterococcus faecalis* hydroxymethylglutaryl-CoA Synthase, mvaS, by Hymeglusin. *Biochemistry* (2012) 51:4713–22. doi: 10.1021/bi300037k
217. Vanommeslaeghe K, MacKerell AD. CHARMM additive and polarizable force fields for biophysics and computer-aided drug design. *Biochim Biophys Acta* (2015) 1850:861–71. doi: 10.1016/j.bbagen.2014.08.004
218. Dror RO, Dirks RM, Grossman JP, Xu H, Shaw DE. Biomolecular simulation: a computational microscope for molecular biology. *Annu Rev Biophys.* (2012) 41:429–52. doi: 10.1146/annurev-biophys-042910-155245
219. Stamler JS, Toone EJ. The decomposition of thionitrites. *Curr Opin Chem Biol.* (2002) 6:779–85. doi: 10.1016/S1367-5931(02)00383-6
220. Hogg N, Singh RJ, Kalyanaraman B. The role of glutathione in the transport and catabolism of nitric oxide. *FEBS Lett.* (1996) 382:223–8.
221. DeMaster EG, Quast BJ, Redfern B, Nagasawa HT. Reaction of nitric oxide with the free sulfhydryl group of human serum albumin yields a sulfenic acid and nitrous oxide. *Biochemistry* (1995) 34:11494–9.
222. Al-Sa'doni HH, Ferro A. S-nitrosothiols as nitric oxide-donors: chemistry, biology and possible future therapeutic applications. *Curr Med Chem.* (2004) 11:2679–90. doi: 10.2174/0929867043364397
223. Zhang Y, Hogg N. S-Nitrosothiols: cellular formation and transport. *Free Radic Biol Med.* (2005) 38:831–8. doi: 10.1016/j.freeradbiomed.2004.12.016
224. Arnelle DR, Stamler JS. NO+, NO, and NO- donation by S-nitrosothiols: implications for regulation of physiological functions by S-nitrosylation and acceleration of disulfide formation. *Arch Biochem Biophys.* (1995) 318:279–85. doi: 10.1006/abbi.1995.1231
225. Rychter M, Gaucher C, Boudier A, Leroy P, Lulek J. S-Nitrosothiols-NO donors regulating cardiovascular cell proliferation: Insight into intracellular pathway alterations. *Int J Biochem Cell Biol.* (2016) 78:156–61. doi: 10.1016/j.biocel.2016.07.003
226. Gaston B, Singel D, Doctor A, Stamler JS. S-nitrosothiol signaling in respiratory biology. *Am J Respir Crit Care Med.* (2006) 173:1186–93. doi: 10.1164/rccm.200510-1584PP
227. Gow AJ, Chen Q, Hess DT, Day BJ, Ischiropoulos H, Stamler JS. Basal and stimulated protein S-nitrosylation in multiple cell types and tissues. *J Biol Chem.* (2002) 277:9637–40. doi: 10.1074/jbc.C100746200
228. Seabra AB, Justo GZ, Haddad PS. State of the art, challenges and perspectives in the design of nitric oxide-releasing polymeric nanomaterials for biomedical applications. *Biotechnol Adv.* (2015) 33:1370–9. doi: 10.1016/j.biotechadv.2015.01.005
229. Seabra AB, Duran N. Nanoparticulated nitric oxide donors and their biomedical applications. *Mini Rev Med Chem.* (2017) 17:216–23. doi: 10.2174/1389557516666160808124624
230. Seabra AB, de Lima R, Calderón M. Nitric oxide releasing nanomaterials for cancer treatment: current status and perspectives. *Curr Top Med Chem.* (2015) 15:298–308. doi: 10.2174/1568026615666150108122918
231. Nakamura T, Lipton S a. Emerging Role of Protein-Protein Transnitrosylation in Cell Signaling Pathways. *Antioxid Redox Signal.* (2012) 18:120709084345003. doi: 10.1089/ars.2012.4703
232. Stamler JS, Toone EJ, Lipton SA, Sucher NJ. (S)NO signals: translocation, regulation, and a consensus motif. *Neuron* (1997) 18:691–6.
233. Moran EE, Timerghazin QK, Kwong E, English AM. Kinetics and mechanism of S-nitrosothiol acid-catalyzed hydrolysis: sulfur activation promotes facile NO+ release. *J Phys Chem B* (2011) 115:3112–26. doi: 10.1021/jp1035597
234. Wolhuter K, Whitwell HJ, Switzer CH, Burgoyne JR, Timms JF, Eaton P. Evidence against Stable Protein S-Nitrosylation as a Widespread Mechanism of Post-translational Regulation. *Mol Cell* (2018) 69:438–50.e5. doi: 10.1016/j.molcel.2017.12.019

235. Sexton DJ, Muruganandam A, McKenney DJ, Mutus B. Visible light photochemical release of nitric oxide from S-nitrosoglutathione: potential photochemotherapeutic applications. *Photochem Photobiol.* (1994) 59:463–7.
236. Singh RJ, Hogg N, Joseph J, Kalyanaraman B. Mechanism of nitric oxide release from S-nitrosothiols. *J Biol Chem.* (1996) 271:18596–603.
237. Lü JM, Wittbrodt JM, Wang K, Wen Z, Schlegel HB, Wang PG, et al. NO affinities of s-nitrosothiols: a direct experimental and computational investigation of RS-NO bond dissociation energies. *J Am Chem Soc.* (2001) 123:2903–4.
238. Bartberger MD, Mannion JD, Powell SC, Stamler JS, Houk KN, Toone EJ. S-N dissociation energies of S-nitrosothiols: on the origins of nitrosothiol decomposition rates. *J Am Chem Soc.* (2001) 123:8868–9. doi: 10.1021/ja0109390
239. de Oliveira* MG, Shishido SM, Seabra AB, Morgon NH. Thermal stability of primary S-Nitrosothiols: roles of autocatalysis and structural effects on the rate of nitric oxide release. *J Phys Chem A* (2002). 106:8963–8970. doi: 10.1021/jp025756u[10.1021/jp025756u]
240. Grossi L, Montevecchi PC. A kinetic study of S-nitrosothiol decomposition. *Chemistry* (2002) 8:380–7. doi: 10.1002/1521-3765(20020118)8:2<380::AID-CHEM380>3.0.CO;2-P
241. Lü J-M, Wittbrodt JM, Wang K, Wen Z, Schlegel HB, Wang PG, et al. NO Affinities of *S* -Nitrosothiols: A direct experimental and computational investigation of RS–NO bond dissociation energies. *J Am Chem Soc* (2001) 123:2903–4. doi: 10.1021/ja000384t
242. Zhelyaskov VR, Gee KR, Godwin DW. Control of NO concentration in solutions of nitrosothiol compounds by light. *Photochem Photobiol.* (1998) 67:282–8.
243. Paunel AN, Dejam A, Thelen S, Kirsch M, Horstjann M, Gharini P, et al. Enzyme-independent nitric oxide formation during UVA challenge of human skin: characterization, molecular sources, and mechanisms. *Free Radic Biol Med.* (2005) 38:606–15. doi: 10.1016/j.freeradbiomed.2004.11.018
244. Mittermayr R, Osipov A, Piskernik C, Haindl S, Dungel P, Weber C, et al. Blue laser light increases perfusion of a skin flap via release of nitric oxide from hemoglobin. *Mol Med.* (2007) 13:22–9. doi: 10.2119/2006-00035.Mittermayr
245. Dungel P, Mittermayr R, Haindl S, Osipov A, Wagner C, Redl H, et al. Illumination with blue light reactivates respiratory activity of mitochondria inhibited by nitric oxide, but not by glycerol trinitrate. *Arch Biochem Biophys.* (2008) 471:109–15. doi: 10.1016/j.abb.2008.01.009
246. Iwamoto Y, Kodera M, Hitomi Y. Uncaging a catalytic hydrogen peroxide generator through the photo-induced release of nitric oxide from a {MnNO}(6) complex. *Chem Commun.* (2015) 51:9539–42. doi: 10.1039/c5cc02566d
247. Akl J, Sasaki I, Lacroix PG, Malfant I, Mallet-Ladeira S, Vicendo P, Farfán N, et al. Comparative photo-release of nitric oxide from isomers of substituted terpyridinenitrosylruthenium(II) complexes: experimental and computational investigations. *Dalton Trans.* (2014) 43:12721–33. doi: 10.1039/c4dt00974f
248. Williams DLH. The Chemistry of S-Nitrosothiols. *Acc Chem Res.* (1999) 32:869–876. doi: 10.1021/ar9800439
249. Williams DL. S-nitrosothiols and role of metal ions in decomposition to nitric oxide. *Methods Enzymol.* (1996) 268:299–308.
250. Brown GC. Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase. *Biochim Biophys Acta* (2001) 1504:46–57.
251. Piantadosi CA. Cardioprotective role of S-nitrosylated hemoglobin from rbc. *J Clin Invest.* (2016) 126:4402–3. doi: 10.1172/JCI91303
252. Scicinski J, Oronsky B, Ning S, Knox S, Peehl D, Kim MM, et al. NO to cancer: the complex and multifaceted role of nitric oxide and the epigenetic nitric oxide donor, RRx-001. *Redox Biol.* (2015) 6:1–8. doi: 10.1016/j.redox.2015.07.002
253. Wink DA, Vodovotz Y, Laval J, Laval F, Dewhirst MW, Mitchell JB. The multifaceted roles of nitric oxide in cancer. *Carcinogenesis* (1998) 19:711–21.
254. Zhang C, Biggs TD, Devarie-Baez NO, Shuang S, Dong C, Xian M. S-Nitrosothiols: chemistry and reactions. *Chem Commun (Camb)* (2017) 53:11266–77. doi: 10.1039/c7cc06574d
255. Dorado JB, Dlugogorski BZ, Kennedy EM, MacKie JC, Gore J, Altarawneh M. Decomposition of S-nitroso species. *RSC Adv.* (2015) 5:29914–23. doi: 10.1039/c5ra03292j
256. Somasundaram V, Basudhar D, Bharadwaj G, No JH, Ridnour LA, Cheng RYS, et al. Molecular mechanisms of nitric oxide in cancer progression, signal transduction, and metabolism. *Antioxid Redox Signal.* (2018) 54:ars.2018.7527. doi: 10.1089/ars.2018.7527
257. Talipov MR, Khomyakov DG, Xian M, Timerghazin QK. Computational design of S-nitrosothiol "click" reactions. *J Comput Chem.* (2013) 34:1527–30. doi: 10.1002/jcc.23279
258. Cardenas AJP, Abelman R, Warren TH. Conversion of nitrite to nitric oxide at zinc via S-nitrosothiols. *Chem Commun.* (2014) 50:168–70. doi: 10.1039/c3cc46102e
259. Bechtold E, King SB. Chemical methods for the direct detection and labeling of S-nitrosothiols. *Antioxid Redox Signal.* (2012) 17:981–91. doi: 10.1089/ars.2012.4570
260. Giles NM, Kumari S, Gang BP, Yuen CWW, Billaud EMF, Giles GI. The molecular design of S-nitrosothiols as photodynamic agents for controlled nitric oxide release. *Chem Biol Drug Des.* (2012) 80:471–8. doi: 10.1111/j.1747-0285.2012.01420.x
261. Zhang Y, Keszler A, Broniowska KA, Hogg N. Characterization and application of the biotin-switch assay for the identification of S-nitrosated proteins. *Free Radic Biol Med.* (2005) 38:874–81. doi: 10.1016/j.freeradbiomed.2004.12.012
262. Broniowska KA, Hogg N. The chemical biology of S-nitrosothiols. *Antioxid Redox Signal.* (2012) 17:969–80. doi: 10.1089/ars.2012.4590
263. Wang K, Zhang W, Xian M, Hou YC, Chen XC, Cheng JP, et al. New chemical and biological aspects of S-nitrosothiols. *Curr Med Chem.* (2000) 7:821–34.
264. Janakiram NB, Rao C V. iNOS-selective inhibitors for cancer prevention: promise and progress. *Future Med Chem.* (2012) 4:2193–204. doi: 10.4155/fmc.12.168
265. Tannous M, Hutnik CM, Tingey DP, Mutus B. S-nitrosoglutathione photolysis as a novel therapy for antifibrosis in filtration surgery. *Invest Ophthalmol Vis Sci.* (2000) 41:749–55.
266. Triggle CR, Hollenberg M, Anderson TJ, Ding H, Jiang Y, Ceroni L, et al. The endothelium in health and disease–a target for therapeutic intervention. *J Smooth Muscle Res.* (2003) 39:249–67. doi: 10.1540/jsmr.39.249
267. Rotta JCG, Lunardi CN, Tedesco AC. Nitric oxide release from the S-nitrosothiol zinc phthalocyanine complex by flash photolysis. *Brazilian J Med Biol Res.* (2003) 36:587–94. doi: 10.1590/S0100-879X2003000500005
268. Hogg N. Biological chemistry and clinical potential of S-nitrosothiols. *Free Radic Biol Med.* (2000) 28:1478–86. doi: 10.1016/S0891-5849(00)00248-3
269. Hunter RA, Schoenfisch MH. *S* -Nitrosothiol analysis via photolysis and amperometric nitric oxide detection in a microfluidic device. *Anal Chem.* (2015) 87:3171–6. doi: 10.1021/ac503220z
270. Richardson G, Benjamin N. Potential therapeutic uses for S-nitrosothiols. *Clin Sci.* (2002) 102:99–105. doi: 10.1042/cs1020099
271. Aranda E, López-Pedrera C, De La Haba-Rodriguez JR, Rodriguez-Ariza A. Nitric oxide and cancer: the emerging role of S-nitrosylation. *Curr Mol Med.* (2012) 12:50–67. doi: 10.2174/156652412798376099

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer MS and handling Editor declared their shared affiliation.

frontiers in Oncology

OPINION
published: 04 September 2018
doi: 10.3389/fonc.2018.00334

Role, Targets and Regulation of (de)nitrosylation in Malignancy

Salvatore Rizza[1]* *and Giuseppe Filomeni*[1,2]*

[1] Redox Signaling and Oxidative Stress Research Group, Cell Stress and Survival Unit, Center for Autophagy, Recycling and Disease, Danish Cancer Society Research Center, Copenhagen, Denmark, [2] Department of Biology, University of Rome Tor Vergata, Rome, Italy

Keywords: ADH5, GSNOR, nitric oxide, NOS, nitrosylation, cancer, FAK1, HIF-1α

NITRIC OXIDE INVOLVEMENT IN CANCER

Nitric oxide (NO) is a free radical that can target cellular biomolecules directly, or by means of the activity of its metabolites (RNS) generated upon reaction with transition metals (e.g., NO^+), oxygen (e.g., N_2O_3), or superoxide ($ONOO^-$). For instance, it is well-documented that NO and RNS affect DNA integrity and mitochondrial physiology, this leading to genetic mutations (1) and damage to the mitochondrial respiratory chain (2, 3), respectively. Processes ranging from apoptosis, angiogenesis, immunity, and neuronal physiology, all show seemingly contradictory behavior in response to NO. Indeed, the relevance of the steady-state NO concentrations represents a key determinant of its biological function. In support to this assumption, it has been demonstrated that cGMP-mediated processes occur at the low n*M* range, whereas higher NO concentrations cause protein kinase B (PKB)/Akt phosphorylation; stabilization of hypoxia inducible factor (HIF)-1α; phosphorylation of p53 and, at the μ*M* range, they can generate detrimental conditions usually referred as to nitrosative stress (**Figure 1**). Likewise, in tumor biology, it is now commonly accepted that high NO concentrations mediate apoptosis and cancer growth inhibition, whereas (relatively) low concentrations usually promote tumor growth and proliferation, this supporting the nature of "doubled-edged sword" molecule for NO (4, 5). This dichotomy originates from the observations that the inducible form of NO synthase (iNOS or NOS2) was implicated in the macrophage-mediated tumor killing process (6, 7) (**Figure 1**). $NOS2^{-/-}$ mice develop intestinal tumors (8), thereby substantiating the protective role of NOS2 within host defense mechanisms (9, 10). In accordance, a growing body of evidence pointed out that NO-releasing drugs can be toxic for cancer cells.

On the other hand, low rate of NO production can promote tumor growth rather than killing. In line with this assumption, the overexpression of NOS isoforms has been detected in a wide range of human tumors. In particular, NOS2 has been found to be upregulated in melanoma, estrogen receptor-negative (ER^-)-breast cancer, as well as in pancreatic, cervical liver and ovarian cancers (10). Moreover, NOS2 seems to be involved in maintaining physiologically relevant levels of NO to sustain the progression phase of carcinogenesis; mainly it is required to promote angiogenesis and to enhance the ability of cancer cells to counteract nutrient paucity in solid tumors and to metastasize (10, 11). NOS2 is also overexpressed in glioma stem cells, and its activity is required for the expression of the cell cycle inhibitor cell division autoantigen-1 (CDA1), which sustains growth and tumorigenicity (12). NOS2 has been also found to be upregulated in hepatocellular carcinoma (HCC), and is often increased in the hepatocytes of patients with chronic hepatitis and alcoholic cirrhosis, conditions that predispose to HCC (13–15). Notwithstanding all these lines of

OPEN ACCESS

Edited by:
Saverio Marchi,
University of Ferrara, Italy

Reviewed by:
Francesco Moccia,
University of Pavia, Italy

***Correspondence:**
Giuseppe Filomeni
giufil@cancer.dk
Salvatore Rizza
rizza@cancer.dk

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *13 June 2018*
Accepted: *02 August 2018*
Published: *04 September 2018*

Citation:
Rizza S and Filomeni G (2018) Role, Targets and Regulation of (de)nitrosylation in Malignancy. Front. Oncol. 8:334. doi: 10.3389/fonc.2018.00334

evidence, investigations on NOS2$^{-/-}$ mice, in spontaneous and fibrosis-associated models of HCC, reveal little effect of NOS2-derived NO on hepatocarcinogenesis (16), meaning that other players are also involved.

S-nitrosylation and Cancer

Redox signal underlying both pro-survival and death pathways, is a molecular information transduced by means of reactive cysteine residues that can undergo *S*-hydroxylation (*SOH*), upon reaction with ROS (i.e., H_2O_2) or *S*-nitrosylation (*SNO*), the posttranslational modification induced by NO, which is now emerging to underlie NO bioactivity (17). In the presence of a sulfhydryl group in their close proximity, both these modifications can resolve in a more stable disulfide bridge (*S*-thiolation, *SS*) (18–20). Actually, it has been recently questioned whether *S*-nitrosylation—given its nature of instable posttranslational modification—is able to convey the NO-mediated signal, or just acts as mere intermediate for disulfide bridge formation (21). Whatever is the end effector (if directly the SNO group or, indirectly, the SS adduct), the extent of *S*-nitrosylation is determined by a delicate balance between: (i) the rate of NO production, which is catalyzed by NOSs (22, 23), (ii) the activity of a recently discovered class of enzymes termed nitrosylases (24, 25), and (iii) the efficiency of SNO removal, that is mediated by denitrosylases. *S*-nitrosoglutathione reductase (GSNOR) represents the prototype of this class of oxidoreductases and, so far, the only denitrosylase able to completely reduce NO moiety, reason why it has been also termed GSNO *terminase* (26–28). Notwithstanding current literature offers still conflicting lines of evidence about the role of NOS/NO system in cancer biology, even less is known on the role played by GSNOR and denitrosylation. In this scenario, it has been reported that GSNOR-ablated (GSNOR-KO) mice show predilection to hepatocellular carcinoma (HCC) in association with *S*-nitrosylation and proteasomal degradation of the DNA damage repair enzyme O^6-alkylguanine-DNA alkyltransferase (AGT) (29). As a result, the repair of carcinogenic O^6-alkylguanines is significantly impaired with a consequence increase in tumorigenesis (29, 30). Analyses performed on human HCC patients showed a significant decrease of GSNOR protein levels and activity in the 50% of cases (30), arguing for a functional link between GSNOR-dependent *S*-nitrosylation and HCC. Although this evidence supports a driving role for GSNOR and excessive *S*-nitrosylation in HCC ontogenesis, it is still unknown whether they are also implicated in the other phases of carcinogenesis, e.g., tumor promotion and progression (31).

In this regard, it has been published that GSNOR deficient HCC cells have a compromised mitochondrial electron transport chain characterized by the upregulation of succinate dehydrogenase (*SDH*), likely as an adapting response to the general impairment of the mitochondrial respiratory machinery (32) derived from excessive nitrosative stress. The hyper-nitrosylation of the mitochondrial chaperone TNF receptor–associated protein 1 (Trap1) has been identified as the molecular event responsible for such a rearrangement and, in turn, for the enhanced sensitivity of GSNOR-downregulating HCC to SDH-targeting mitochondrial drugs (32). Nevertheless, it is worth to note that the mean size of GSNOR-deficient tumor xenografts is larger (approximately the double) than parental (GSNOR-proficient) HCC (32), suggesting that excessive *S*-nitrosylation arising from GSNOR loss, might promote tumor progression and growth *in vivo*. This hypothesis finds support in a recent study correlating GSNOR downregulation with HER2$^+$ breast cancer resistance to trastuzumab and poor patient prognosis (33). Altogether, these pieces of evidence argue for a new role of GSNOR in malignancy and resistant phenotypes of breast cancer. However, no evidence about the molecular mechanisms underneath has been provided so far.

Known and Supposed Targets of *S*-nitrosylation in Aggressive Cancer

Based on what above reported, it is plausible that impairments of denitrosylation capacity (e.g., upon GSNOR deficiency) modulates the function/activity of oncoproteins susceptible to *S*-nitrosylation. These defects, more specifically than a general increase of NO production (that could impact on a plethora of different targets and to a different extent) might account for a deregulated NO-signaling in carcinogenesis. This hypothesis is further sustained by a very recent study indicating that GSNOR-deficiency (and excessive *S*-nitrosylation deriving from it), is a condition associated with aging (34, 35), which represents a major risk factor for cancer development. Actually, cancer might count as an aging disease, and shares with aging some common features (e.g., genomic instability, telomere shortening, oxidative stress, deregulation of nutrient sensing) that, indeed, characterize both disorders (36).

Besides those previously mentioned, and others well documented to play a role in apoptosis (e.g., p53, Bcl2, and Fas), many oncoproteins have been discovered in the last decades to undergo *S*-nitrosylation. The modification of oncoproteins and tumor suppressors by NO—independently on the effects induced, whether gain- or loss-of-function (23, 37)—is emerging as a critical phenomenon associated with neoplastic transformation. Some of these NO-modified oncoproteins participate to signal transduction and are found mutated or modulated in cancer. Within this class of proteins in which *S*-nitrosylation has been identified as pro-oncogenic modification, we can list: (i) the GTPase Ras (nitrosylated at Cys118) (23, 38), which underlies cancer cell growth downstream of receptor-associated tyrosine kinases; (ii) the phosphatase and tensin homolog PTEN (nitrosylated at Cys83) (39), which regulates the levels of phosphatidyl inositole-3-phosphate/Akt-dependent pathway; (iii) the protein kinase c-Src, which represents one of the master regulators of tumor proliferation, invasion and metastatic phenotype, and has been found to be nitrosylated at Cys498 (40) (**Figure 1**). Interestingly, this residue is conserved throughout the Src family of protein tyrosine kinases (SFKs) and, at least for other two members, i.e., Yes and Fyn, has been also reported to stimulate their activation (40). Focal adhesion kinase 1 (FAK1) is also comprised in the

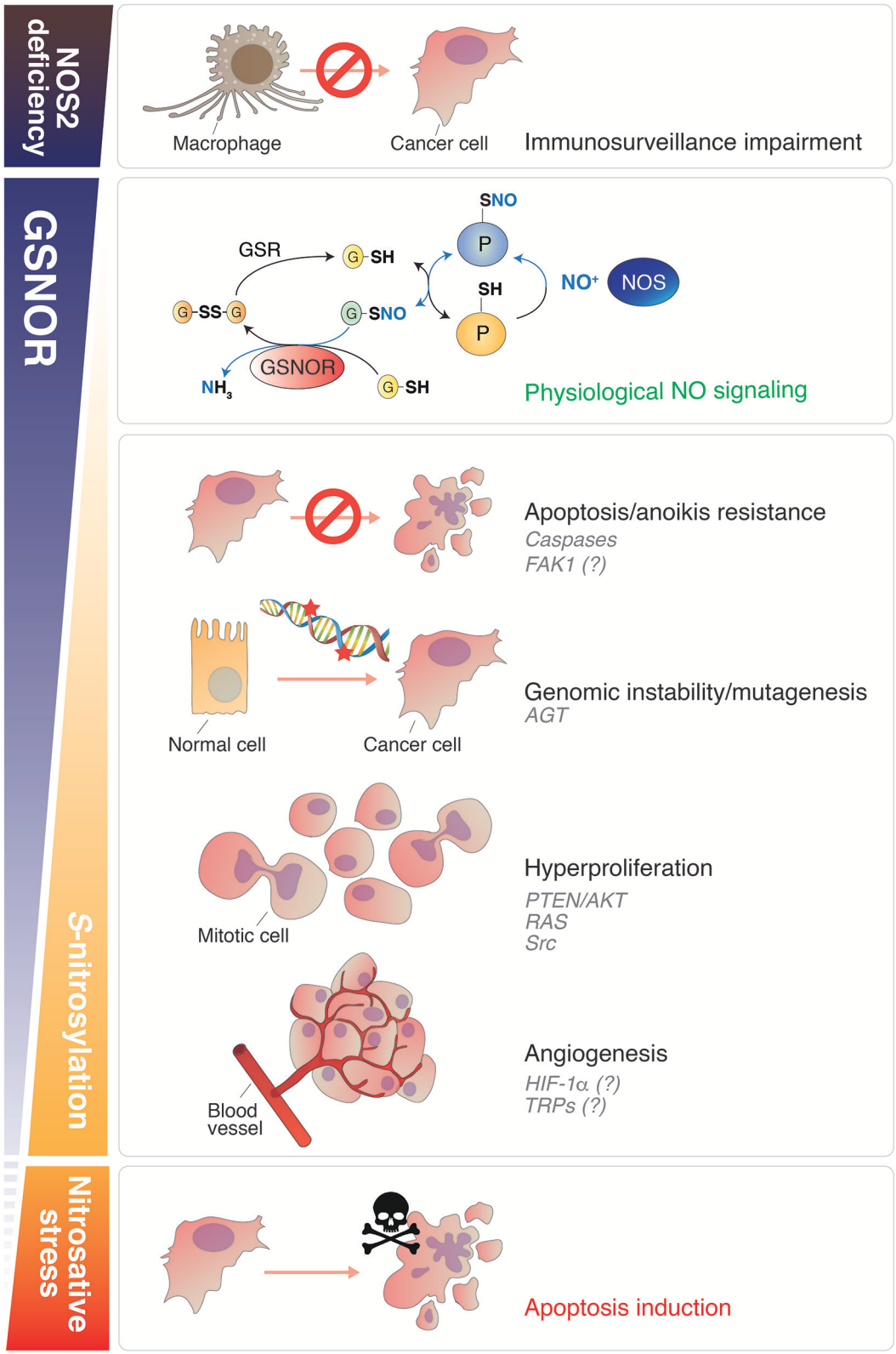


FIGURE 1 | Roles of NO signaling and protein denitrosylation in cancer. Nitric oxide plays different roles in cancer biology depending on its concentration. GSNOR is the main cellular denitrosylase. Counteracting the effects induced by NOS, GSNOR finely modulates protein *S*-nitrosylation (second panel from the top), which is establishing as the main posttranslational modification underlying NO bioactivity. A disbalance in NO signaling can promote tumor induction, survival and progression.
(Continued)

FIGURE 1 | NOS2 deficiency impairs the capability of macrophages to kill cancer cells (Top). Conversely, in conditions of normal (or induced) NOS activity, GSNOR decrease has been linked to many cancer hallmarks, such as: (i) apoptosis and anoikis resistance (due to caspases and, reasonably, FAK-1 *S*-nitrosylation); (ii) genomic instability (DNA repair impairment, due to AGT *S*-nitrosylation and degradation); (iii) cells hyperproliferation (*via* the NO-mediated activation of oncoproteins, such as AKT, RAS, and Src); (iv) angiogenesis (putatively regulated by HIF-1α and TRPs *S*-nitrosylation). Extreme nitrosative stress conditions—induced, for instance, by NOS overexpression or by the use of NO-donors—activate cell death and are implemented (or physiologically activated in macrophages) to destroy cancer cells (Bottom). NO, nitric oxide; GSNOR, *S*-nitrosoglutathione reductase; NOS, nitric oxide synthase; FAK1, focal adhesion kinase 1; AGT, O^6-methylguanine-DNA methyltransferase; HIF-1α, hypoxia-inducible factor-1α; TRP, Transient receptor potential channel.

SFKs family. It is phosphorylated upon integrin engagement in a Src-dependent or independent (auto-phosphorylation) fashion, thus initiating multiple downstream signaling pathways responsible for aggressive and metastatic phenotype (e.g., resistance to anoikis and cell migration) (41). Similarly to Src, Yes and Fyn have been reported to act as FAK1-interacting kinases and to be involved in FAK1 activation as well (42, 43). Notwithstanding this tight relationship, triple-KO cells in which Src, Yes, and Fyn expression is suppressed (*SYF* cells), still show phospho-active levels of FAK1 upon treatment with NO donors (40). This unexpected evidence clearly indicates that *S*-nitrosylation of Src, Yes, and Fyn is dispensable for NO-driven phosphorylation of FAK1 and, interestingly, suggests that FAK1 might represent a direct target of *S*-nitrosylation (**Figure 1**), with this modification driving its oncogenic function.

Another oncoprotein, which has been identified to be crucial in cancer cell survival and growth, especially under low-oxygen tension (hypoxia), is HIF-1α. HIF-1α deregulation has been deeply implicated in different aspects of cancer biology, such as angiogenesis, cell resistance, and tumor invasion (44–47). From a metabolic point of view, HIF-1α aberrant activation underpins the so-called "Warburg effect": the preferential glycolytic consumption of glucose in cancer cells, which takes place also under normal oxygen tension. HIF1α has been found nitrosylated at Cys533 (48), with this being relevant in stroke and cardiovascular disease. However, if S-nitrosylation might somehow induce HIF1α oncogenic activity still remains neglected (**Figure 1**) and would deserve to be investigated in the future.

Among the various classes of proteins that have been identified in the last decades as being activated by *S*-nitrosylation, the transient receptor potential (TRP) ion channels (49), which represent a huge family of proteins underpinning, among others, warm, taste, and pain sensory transduction, are worth to be mentioned. Besides their well-documented role in the nervous system as mediators of sensations, in the last years it is emerging that many TRPs, such as those belonging to the "melastatin" (TRPM), "vanilloid" (TRPV), and "ankyrin" (TRPA) subfamilies, are overexpressed in many cancer types, this being pivotal for calcium signaling-dependent control of tumor-promoting processes, e.g., vascularization and metastasis (50, 51). In particular, it has been proposed that, by modulating intracellular Ca^{2+} concentrations, TRPs are deeply involved in tumor initiation, progression and resistance (52). In this context, it has been very recently found out that TRPA1 is upregulated in breast and lung cancer downstream of the activation of Nrf2, the master regulator of antioxidant response, this conferring non-canonical resistance to tumor cells against oxidative stress and ROS-producing chemotherapeutics (53). Many other observations argue for TRPs inhibition being a promising tool to eradicate cancer (54–56). However, notwithstanding the evidence that *S*-nitrosylation interferes with TRPs activity and calcium signaling, to date there's still no indication supporting a direct involvement of TRPs *S*-nitrosylation in carcinogenesis (**Figure 1**). Mostly, there's still no study aimed at understanding whether TRPs targeting on nitrosylable cysteines might represent a novel line of intervention in cancer treatment.

Putative Mechanisms That Affect Denitrosylation in Cancer

The above reported evidence points out that defects in GSNOR expression and denitrosylation are pivotal for sustaining the tumorigenic effects of NO, namely, its role in the progression phase of cancer (31). A recent report on the epigenetic regulation of GSNOR might be of help to understand how this condition can be established in cancer. In particular, it has been demonstrated that *GSNOR* expression is controlled by the activity of the demethylase Ten-eleven translocation protein 1 (Tet1), a member of the 2-oxoglutarate–dependent dioxygenases that regulates transcription by removing methyl groups from CpG islands located in the promoter regions of genes (34). Remarkably, Tet1 expression has been found to be reduced in a wide range of solid cancers, such as melanoma, prostate, lung, and liver tumors (57, 58)—where also GSNOR mRNA seems to be downregulated—and to correlate with advanced cancer stage, nodal metastases, and poor survival rate in breast cancer patients (59). These lines of evidence suggest that GSNOR might be epigenetically downregulated in aggressive cancer as a consequence of Tet1 reduction, thus providing a new link between epigenetics and redox signaling. This hypothesis can be even extended to further mechanisms of epigenetic regulation. Indeed, given the complex structure of GSNOR mRNA, it has been proposed that GSNOR expression might be also regulated *via* microRNAs (miRs) (27). However, no putative miRs, able to target GSNOR transcript, has been so far identified to be upregulated in cancer, or hypothesized acting as additional modulators of *S*-nitrosylation.

CONCLUSION

The role of GSNOR-mediated denitrosylation in carcinogenesis has been capturing the interest of many researchers working on cancer biology, as many lines of evidence indicate that this

process is frequently deregulated in cancer cells. In this article, we have tried to summarize what has been discovered in the last years and provide some hints on possible aspects that are still overlooked. Understanding how GSNOR expression is deregulated in may cancer histotypes, as well as the mechanisms underlying the modification of new protein targets involved in cancer resistance and aggressiveness, are, indeed, issues that deserve to be investigated in the future, since they could set the stage for new anticancer approaches interfering with the redox adaptation distinctive of many cancer cells.

AUTHOR CONTRIBUTIONS

GF conceived the paper. GF and SR wrote the paper. SR drew the figure.

ACKNOWLEDGMENTS

This work has been supported by Danish Cancer Society Grant KBVU R146-A9414, and Associazione Italiana per la Ricerca sul Cancro (AIRC) Grant IG20719.

REFERENCES

1. Dizdaroglu M, Jaruga P. Mechanisms of free radical-induced damage to DNA. *Free Radic Res.* (2012) 46:382–419. doi: 10.3109/10715762.2011.653969
2. Boveris A, Costa LE, Poderoso JJ, Carreras MC, Cadenas E. Regulation of mitochondrial respiration by oxygen and nitric oxide. *Ann NY Acad Sci.* (2006) 899:121–35. doi: 10.1111/j.1749-6632.2000.tb06181.x
3. Brown GC, Borutaite V. Nitric oxide inhibition of mitochondrial respiration and its role in cell death. *Free Radic Biol Med.* (2002) 33:1440–50. doi: 10.1016/S0891-5849(02)01112-7
4. Burke AJ, Sullivan FJ, Giles FJ, Glynn SA. The yin and yang of nitric oxide in cancer progression. *Carcinogenesis* (2013) 34:503–12. doi: 10.1093/carcin/bgt034
5. Vanini F, Kashfi K, Nath N. The dual role of iNOS in cancer. *Redox Biol.* (2015) 6:334–43. doi: 10.1016/j.redox.2015.08.009
6. Marletta MA. Nitric oxide synthase: aspects concerning structure and catalysis. *Cell* (1994) 78:927–30. doi: 10.1016/0092-8674(94)90268-2
7. Bogdan C. Nitric oxide and the immune response. *Nat Immunol.* (2001) 2:907–16. doi: 10.1038/ni1001-907
8. Yerushalmi HF, Besselsen DG, Ignatenko NA, Blohm-Mangone KA, Padilla-Torres JL, Stringer DE, et al. The role of NO synthases in arginine-dependent small intestinal and colonic carcinogenesis. *Mol Carcinog.* (2006) 45:93–105. doi: 10.1002/mc.20168
9. Xu W, Liu LZ, Loizidou M, Ahmed M, Charles IG. The role of nitric oxide in cancer. *Cell Res.* (2002) 12:311–20. doi: 10.1038/sj.cr.7290133
10. Thomas DD, Wink DA. NOS2 as an emergent player in progression of cancer. *Antioxid Redox Signal* (2017) 26:963–5. doi: 10.1089/ars.2016.6835
11. Jadeski LC, Hum KO, Chakraborty C, Lala PK. Nitric oxide promotes murine mammary tumour growth and metastasis by stimulating tumour cell migration, invasiveness and angiogenesis. *Int J Cancer* (2000) 86:30–9. doi: 10.1002/(SICI)1097-0215(20000401)86:1<30::AID-IJC5>3.0.CO;2-I
12. Eyler CE, Wu Q, Yan K, MacSwords JM, Chandler-Militello D, Misuraca KL, et al. Glioma stem cell proliferation and tumor growth are promoted by nitric oxide synthase-2. *Cell* (2011) 146:53–66. doi: 10.1016/j.cell.2011.06.006
13. Majano PL, García-monzón C, López-cabrera M, Lara-pezzi E, Fernández-ruiz E, García-iglesias C, et al. Inducible Nitric oxide synthase expression in chronic viral Hepatitis. evidence for a virus-induced gene upregulation. *J Clin Invest.* (1998) 101:1343-52.
14. Kane JM, Shears LL, Hierholzer C, Ambs S, Billiar TR, Posner MC. Chronic hepatitis C virus infection in humans: induction of hepatic nitric oxide synthase and proposed mechanisms for carcinogenesis. *J Surg Res.* (1997) 69:321–4. doi: 10.1006/jsre.1997.5057
15. McNaughton L, Puttagunta L, Martinez-Cuesta MA, Kneteman N, Mayers I, Moqbel R, et al. Distribution of nitric oxide synthase in normal and cirrhotic human liver. *Proc Natl Acad Sci USA. (2002)* 99:17161–6. doi: 10.1073/pnas.0134112100
16. Tang CH, Wei W, Hanes MA, Liu L. Hepatocarcinogenesis driven by GSNOR deficiency is prevented by iNOS inhibition. *Cancer Res.* (2013) 73:2897–904. doi: 10.1158/0008-5472.CAN-12-3980
17. Stamler JS, Lamas S, Fang FC. Nitrosylation: the prototypic redox-based signaling mechanism. *Cell* (2001) 106:675–83. doi: 10.1016/S0092-8674(01)00495-0
18. Sengupta R, Holmgren A. Thioredoxin and thioredoxin reductase in relation to reversible *S* -Nitrosylation. *Antioxid Redox Signal* (2013) 18:259–69. doi: 10.1089/ars.2012.4716
19. Martínez-Ruiz A, Araújo IM, Izquierdo-Álvarez A, Hernansanz-Agustín P, Lamas S, Serrador JM. Specificity in S-Nitrosylation: a short-range mechanism for NO signaling? *Antioxid Redox Signal* (2013) 19:1220–35. doi: 10.1089/ars.2012.5066
20. Jones DP, Sies H. The Redox Code. *Antioxid Redox Signal* (2015) 23: 734–46. doi: 10.1089/ars.2015.6247
21. Wolhuter K, Whitwell HJ, Switzer CH, Burgoyne JR, Timms JF, Eaton P. Evidence against stable protein S-Nitrosylation as a Widespread mechanism of post-translational regulation. *Mol Cell* (2018) 69:438–50.e5. doi: 10.1016/j.molcel.2017.12.019
22. Hess DT, Stamler JS. Regulation by S-Nitrosylation of protein post-translational modification. *J Biol Chem.* (2012) 287:4411–8. doi: 10.1074/jbc.R111.285742
23. Hess DT, Matsumoto A, Kim S-OO, Marshall HE, Stamler JS. Protein S-Nitrosylation: purview and parameters. *Nat Rev Mol Cell Biol.* (2005) 6:150–66. doi: 10.1038/nrm1569
24. Seth D, Hess DT, Hausladen A, Wang L, Wang Y, Stamler JS. A multiplex enzymatic machinery for cellular protein S-Nitrosylation. *Mol Cell* (2018) 69:451–64.e6. doi: 10.1016/j.molcel.2017.12.025
25. Jia J, Arif A, Terenzi F, Willard B, Plow EF, Hazen SL, et al. Target-selective protein S-Nitrosylation by sequence motif recognition. *Cell* (2014) 159:623–34. doi: 10.1016/j.cell.2014.09.032
26. Liu L, Hausladen A, Zeng M, Que L, Heitman J, Stamler JS. A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. *Nature* (2001) 410:490–4. doi: 10.1038/35068596
27. Rizza S, Filomeni G. Chronicles of a reductase: biochemistry, genetics and physio-pathological role of GSNOR. *Free Radic Biol Med.* (2017) 110:19–30. doi: 10.1016/j.freeradbiomed.2017.05.014
28. Jensen DE, Belka GK, Du Bois GC. S-Nitrosoglutathione is a substrate for rat alcohol dehydrogenase class III isoenzyme. *Biochem J.* (1998) 331(Pt. 2):659–68.
29. Wei W, Yang Z, Tang CH, Liu L. Targeted deletion of GSNOR in hepatocytes of mice causes nitrosative inactivation of O6-alkylguanine-dna alkyltransferase and increased sensitivity to genotoxic diethylnitrosamine. *Carcinogenesis* (2011) 32:973–7. doi: 10.1093/carcin/bgr041
30. Wei W, Li B, Hanes MA, Kakar S, Chen X, Liu L, et al. S-Nitrosylation from GSNOR deficiency impairs DNA repair and promotes hepatocarcinogenesis. *Sci Transl Med.* (2010) 2:19ra13. doi: 10.1126/scitranslmed.3000328
31. Rizza S, Filomeni G. Tumor suppressor roles of the denitrosylase GSNOR. *Crit Rev Oncog.* (2016) 21:433–45. doi: 10.1615/CritRevOncog.2017021074
32. Rizza S, Montagna C, Cardaci S, Maiani E, Di Giacomo G, Sanchez-Quiles V, et al. S-Nitrosylation of the mitochondrial chaperone TRAP1 sensitizes hepatocellular carcinoma cells to inhibitors of succinate dehydrogenase. *Cancer Res.* (2016) 76:4170–82. doi: 10.1158/0008-5472.CAN-15-2637
33. Cañas A, López-Sánchez LM, Peñarando J, Valverde A, Conde F, Hernández V, et al. Altered S-nitrosothiol homeostasis provides a survival advantage to breast cancer cells in HER2 tumors and reduces their sensitivity to trastuzumab. *Biochim Biophys Acta* (2016) 1862:601–10. doi: 10.1016/j.bbadis.2016.02.005

34. Rizza S, Cardaci S, Montagna C, Di Giacomo G, De Zio D, Bordi M, et al. S -Nitrosylation drives cell senescence and aging in mammals by controlling mitochondrial dynamics and mitophagy. *Proc Natl Acad Sci USA*. (2018) 21:7–13. doi: 10.1073/pnas.1722452115
35. Rizza S, Filomeni G. Denitrosylate and live longer: how GSNOR links mitophagy to aging. *Autophagy* (2018) 20:1–3. doi: 10.1080/15548627.2018.1475818
36. Aunan JR, Cho WC, Søreide K. The biology of aging and cancer: a brief overview of shared and divergent molecular hallmarks. *Aging Dis.* (2017) 8:628–42. doi: 10.14336/AD.2017.0103
37. Wang Z. Protein S-Nitrosylation and cancer. *Cancer Lett.* (2012) 320:123–9. doi: 10.1016/j.canlet.2012.03.009
38. Heo J, Campbell SL. Mechanism of p21Ras S-NITROSYLATION and kinetics of nitric oxide-mediated guanine nucleotide exchange. *Biochemistry* (2004) 43:2314–22. doi: 10.1021/bi035275g
39. Numajiri N, Takasawa K, Nishiya T, Tanaka H, Ohno K, Hayakawa W, et al. On-off system for PI3-kinase-Akt signaling through S-Nitrosylation of phosphatase with sequence homology to tensin (PTEN). *Proc Natl Acad Sci USA*. (2011) 108:10349–54. doi: 10.1073/pnas.1103503108
40. Rahman MA, Senga T, Ito S, Hyodo T, Hasegawa H, Hamaguchi M. S-Nitrosylation at cysteine 498 of c-Src tyrosine kinase regulates nitric oxide-mediated cell invasion. *J Biol Chem.* (2010) 285:3806–14. doi: 10.1074/jbc.M109.059782
41. Sulzmaier FJ, Jean C, Schlaepfer DD. FAK in cancer: mechanistic findings and clinical applications. *Nat Rev Cancer* (2014) 14:598–610. doi: 10.1038/nrc3792
42. Hamamura K, Tsuji M, Hotta H, Ohkawa Y, Takahashi M, Shibuya H, et al. Functional activation of Src family kinase yes protein is essential for the enhanced malignant properties of human melanoma cells expressing ganglioside GD3. *J Biol Chem.* (2011) 286:18526–37. doi: 10.1074/jbc.M110.164798
43. Sen B, Johnson FM. Regulation of Src family kinases in human cancers. *J Signal Transduct.* (2011) 2011:1–14. doi: 10.1155/2011/865819
44. Unwith S, Zhao H, Hennah L, Ma D. The potential role of HIF on tumour progression and dissemination. *Int J Cancer* (2015) 136:2491–503. doi: 10.1002/ijc.28889
45. Semenza GL. HIF-1 mediates metabolic responses to intratumoral hypoxia and oncogenic mutations. *J Clin Invest.* (2013) 123:3664–71. doi: 10.1172/JCI67230
46. Rankin EB, Giaccia A J. The role of hypoxia-inducible factors in tumorigenesis. *Cell Death Differ.* (2008) 15:678–85. doi: 10.1038/cdd.2008.21
47. Wu L, Fu Z, Zhou S, Gong J, Liu CA, Qiao Z, et al. HIF-1α and HIF-2α: Siblings in promoting angiogenesis of residual hepatocellular carcinoma after high-intensity focused ultrasound ablation. *PLoS ONE* (2014) 9:1–10. doi: 10.1371/journal.pone.0088913
48. Li F, Sonveaux P, Rabbani ZN, Liu S, Yan B, Huang Q, et al. Regulation of HIF-1α Stability through S-Nitrosylation. *Mol Cell* (2007) 26:63–74. doi: 10.1016/j.molcel.2007.02.024
49. Yoshida T, Inoue R, Morii T, Takahashi N, Yamamoto S, Hara Y, et al. Nitric oxide activates TRP channels by cysteine S-Nitrosylation. *Nat Chem Biol.* (2006) 2:596–607. doi: 10.1038/nchembio821
50. Fels B, Bulk E, Petho Z, Schwab A. The role of TRP channels in the metastatic cascade. *Pharmaceuticals* (2018) 11:E48. doi: 10.3390/ph11020048
51. Pla AF, Gkika D. Emerging role of TRP channels in cell migration: from tumor vascularization to metastasis. *Front Physiol.* (2013) 4:311. doi: 10.3389/fphys.2013.00311
52. Fliniaux I, Germain E, Farfariello V, Prevarskaya N. TRPs and Ca^{2+} in cell death and survival. *Cell Calcium* (2018) 69:4–18. doi: 10.1016/j.ceca.2017.07.002
53. Reczek CR, Chandel NS. ROS promotes cancer cell survival through calcium signaling. *Cancer Cell* (2018) 33:949–51. doi: 10.1016/j.ccell.2018.05.010
54. Almasi S, Kennedy BE, El-Aghil M, Sterea AM, Gujar S, Partida-Sánchez S, et al. TRPM2 channel–mediated regulation of autophagy maintains mitochondrial function and promotes gastric cancer cell survival via the JNK-signaling pathway. *J Biol Chem.* (2018) 293:3637–50. doi: 10.1074/jbc.M117.817635
55. Bao L, Chen S, Conrad K, Keefer K, Abraham T, Lee JP, et al. Depletion of the human ion channel TRPM2 in neuroblastoma demonstrates its key role in cell survival through modulation of mitochondrial reactive oxygen species and bioenergetics. *J Biol Chem.* (2016) 291:24449–64. doi: 10.1074/jbc.M116.747147
56. Singh AK, Saotome K, McGoldrick LL, Sobolevsky AI. Structural bases of TRP channel TRPV6 allosteric modulation by 2-APB. *Nat Commun.* (2018) 9:2465. doi: 10.1038/s41467-018-04828-y
57. Liu C, Liu L, Chen X, Shen J, Shan J, Xu Y, et al. Decrease of 5-hydroxymethylcytosine is associated with progression of hepatocellular carcinoma through downregulation of TET1. *PLoS ONE* (2013) 8:e62828. doi: 10.1371/journal.pone.0062828
58. Yang H, Liu Y, Bai F, Zhang JY, Ma SH, Liu J, et al. Tumor development is associated with decrease of TET gene expression and 5-methylcytosine hydroxylation. *Oncogene* (2013) 32:663–9. doi: 10.1038/onc.2012.67
59. Hsu CH, Peng KL, Kang ML, Chen YR, Yang YC, Tsai CH, et al. TET1 suppresses cancer invasion by activating the tissue inhibitors of metalloproteinases. *Cell Rep.* (2012) 2:568–79. doi: 10.1016/j.celrep.2012.08.030

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 25 May 2018
doi: 10.3389/fonc.2018.00170

The Mitochondrial Citrate Carrier (SLC25A1) Sustains Redox Homeostasis and Mitochondrial Metabolism Supporting Radioresistance of Cancer Cells With Tolerance to Cycling Severe Hypoxia

*Julian Hlouschek, Christine Hansel, Verena Jendrossek and Johann Matschke**

Institute of Cell Biology (Cancer Research), University Hospital Essen, University of Duisburg-Essen, Essen, Germany

Pronounced resistance of lung cancer cells to radiotherapy and chemotherapy is a major barrier to successful treatment. Herein, both tumor hypoxia and the upregulation of the cellular antioxidant defense systems observed during malignant progression can contribute to radioresistance. We recently found that exposure to chronic cycling severe hypoxia/reoxygenation stress results in glutamine-dependent upregulation of cellular glutathione (GSH) levels and associated radiation resistance opening novel routes for tumor cell-specific radiosensitization. Here, we explored the role of the mitochondrial citrate carrier (SLC25A1) for the improved antioxidant defense of cancer cells with tolerance to acute and chronic severe hypoxia/reoxygenation stress and the use of pharmacologic SLC25A1 inhibition for tumor cell radiosensitization. Exposure to acute or chronic cycling severe hypoxia/reoxygenation stress triggered upregulated expression of SLC25A1 in lung cancer, prostate cancer, and glioblastoma cells *in vitro*. Interestingly, exposure to ionizing radiation (IR) further promoted SLC25A1 expression. Inhibition of SLC25A1 by 1,2,3-benzene-tricarboxylic acid (BTA) disturbed cellular and mitochondrial redox homeostasis, lowered mitochondrial metabolism, and reduced metabolic flexibility of cancer cells. Even more important, combining IR with BTA was able to overcome increased radioresistance induced by adaptation to chronic cycling severe hypoxia/reoxygenation stress. This radiosensitizing effect of BTA-treated cells was linked to increased reactive oxygen species and reduced DNA repair capacity. Of note, key findings could be reproduced when using the SLC25A1-inhibitor 4-Chloro-3-[[(3-nitrophenyl)amino]sulfonyl]-benzoic acid (CNASB). Moreover, *in silico* analysis of publically available databases applying the Kaplan–Meier plotter tool (kmplot.com) revealed that overexpression of SLC25A1 was associated with reduced survival of lung cancer patients suggesting a potential link to aggressive cancers. We show that SLC25A1 can contribute to the increased antioxidant defense of cancer cells allowing them to escape the cytotoxic effects of IR. Since upregulation of SLC25A1 is induced by adverse conditions in the tumor environment, exposure to IR, or both pharmacologic inhibition of SLC25A1 might be an effective strategy for radiosensitization of cancer cells particularly in chronically hypoxic tumor fractions.

Keywords: redox homeostasis, SLC25A1, radiation resistance, chronic hypoxia, cell metabolism, mitochondria, DNA repair

OPEN ACCESS

Edited by:
Andrea Rasola,
Università degli Studi di Padova, Italy

Reviewed by:
Alessandra Castegna,
University of Bari Medical School, Italy
Margaret Ashcroft,
University of Cambridge, United Kingdom

***Correspondence:**
Johann Matschke
johann.matschke@uk-essen.de

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *22 February 2018*
Accepted: *01 May 2018*
Published: *25 May 2018*

Citation:
Hlouschek J, Hansel C, Jendrossek V and Matschke J (2018) The Mitochondrial Citrate Carrier (SLC25A1) Sustains Redox Homeostasis and Mitochondrial Metabolism Supporting Radioresistance of Cancer Cells With Tolerance to Cycling Severe Hypoxia. Front. Oncol. 8:170. doi: 10.3389/fonc.2018.00170

INTRODUCTION

More than 50% of NSCLC patients receive radiotherapy (RT) or radiochemotherapy (RCT) as part of their treatment. Recent meta-analysis revealed that tumor hypoxia is major biological barrier to successful chemotherapy, RT, and potentially some targeted therapies, promoting treatment failure and poor prognosis of patients suffering from non-small cell lung cancer (NSCLC) (1). Though targeting hypoxia-mediated therapy resistance is considered as an attractive approach to improve therapy outcome in solid human tumors including NSCLC, so far clinical trials evaluating the use of hypoxia-targeting agents did not meet the expectations as they failed to reveal a benefit for the patients (1). This might at least be partially due to the lack of appropriate predictive biomarkers for patient selection but emphasizes the need for the definition of mechanism-based more effective novel therapeutic strategies to overcome hypoxia-induced therapy resistance and the co-development of predictive biomarkers and improved imaging of heterogeneous tumor hypoxia to guide RT protocols (1).

Tumors form a complex microenvironment by co-opting various normal tissue cells and immune cells to support their growth and survival (2). However, the imbalance between cell growth and tumor vascularization limits not only the availability of nutrients and oxygen (O_2) but also the removal of secreted potentially toxic metabolites such as lactic acid. As a consequence of rapid proliferation, poor blood supply and altered metabolism tumor hypoxia is frequently linked to lactic acidosis and thus a low pH in the tumor (3, 4). The resulting O_2-deprived and nutrient-deprived acidic microenvironment exerts a selection pressure on the tumor cells thereby directing the acquisition of genetic and epigenetic alterations that allow the cancer cells to survive and to adapt to these adverse conditions during multistep carcinogenesis thereby promoting tumor growth and even metastasis (5–8). Moreover, accumulating evidence indicates that the adverse microenvironment also impacts the therapy response at multiple levels and promotes the resistance of solid tumors to chemotherapy and RT (9, 10).

Herein limited availability of O_2 known as "tumor hypoxia" is considered as a major environmental factor driving genomic instability, malignant progression, and resistance of solid tumors to RT and chemotherapy (9–11). The cytotoxic efficacy of RT and certain DNA-damaging anticancer drugs relies on the formation of reactive oxygen species (ROS) and thus on local availability of molecular O_2 in the tumor tissue during treatment delivery; therefore, an acute decrease in O_2 levels as a consequence of insufficient O_2 supply confers direct resistance by decreasing oxidative stress and therapy-induced cell killing, the so-called "oxygen-effect" (10, 12, 13). Moreover, cancer cells dispose of multiple survival pathways that allow them to adapt to acute hypoxia and survive these adverse conditions [for a review, see Ref. (14)].

But tumor hypoxia is highly dynamic with respect to its duration (short term to long term) and schedule (transient, chronic, or intermittent), and also fluctuates regionally presumably as a result of the instability and chaotic organization of the tumor vasculature (15–18). Thus, considerable fractions of human vascularized solid tumors are exposed to dynamic changes between hypoxia and intermittent reoxygenation. Chronic changes between hypoxia and intermittent reoxygenation ("cycling hypoxia") constitute a major driving force in the development of malignant progression, tumor heterogeneity, and clonal evolution of therapy-resistant cells (15, 19–24). Acute hypoxia/reoxygenation stress and tumor cell adaptation to chronic cycling severe hypoxia/reoxygenation stress also impact the outcome of cancer RT (23–26). A detailed understanding of the processes that allow cancer cells to escape the cytotoxic effects of RT in the adverse tumor microenvironment is required if we aim to develop effective therapeutic strategies to improve therapy outcome.

In this context, others and we demonstrated that adaptation to chronic hypoxia/reoxygenation stress drives upregulation of cellular GSH levels to avoid excessive ROS damage and promote death resistance (24, 27, 28). Altered nutrient and energy metabolism is one of the emerging hallmarks of cancer cells (29, 30). Moreover, a progressive upregulation of cellular antioxidant systems has been associated with malignant progression (31), suggesting a broader relevance of the above findings for understanding tumor progression and clonal evolution of therapy-resistant cells in tumors with heterogeneous environments. Importantly, as a proof of principle others and we demonstrated that targeting metabolic reprogramming associated with increased GSH levels is a promising strategy for radiosensitization (24, 32).

However, the above studies also demonstrated that cancer cells use different metabolic adaptation strategies to increase their cellular GSH levels, avoid ROS-dependent damage, and escape genotoxic therapies, presumably depending on the genetic background (24, 28, 31–33). Thus, targeting altered glutamine usage will be a viable therapeutic strategy to reduce glutathione levels in some but not all cancer models (24). We therefore reasoned that targeting the increased GSH-based antioxidant capacity and thus the common phenotype of aggressive cancer cells with increased stress tolerance, might be an effective strategy for therapeutic intervention with broader relevance.

Generally, increased GSH levels can be targeted by using drugs interfering with the regeneration of glutathione, the provision of reduction equivalents, increased glutathione synthesis, or glutathione transport and uptake (24, 34, 35). In an effort to define novel ways to specifically target increased GSH levels in aggressive cancer cells the observation about a link between the mitochondrial citrate carrier (CIC, also known as mitochondrial citrate transport protein, CTP) and the maintenance of cytosolic and mitochondrial NADPH pools and the mitochondrial redox homeostasis attracted our attention (36, 37).

The CIC is encoded by the *SLC25A1* gene located on chromosome 22q11.2. Besides citrate, SLC25A1 is also responsible for the electroneutral transport of isocitrate, malate, and phosphoenolpyruvate (38). Furthermore, SLC25A1—together with cytosolic isocitrate dehydrogenase 1 (IDH1) and mitochondrial isocitrate dehydrogenase 2 (IDH2)—takes part in the transport of NADPH derived from reductive carboxylation over the mitochondrial membrane (36) and might thus play a role in GSH regeneration. Overall, SLC25A1 is important for the maintenance of mitochondrial homeostasis and its overexpression was shown to drive tumorigenesis in various types of cancer (39).

Though the authors linked SLC25A1 expression to anchorage-independent growth of NCI-H460 cancer cells (36), it was tempting to speculate that the function of SLC25A1 regarding maintenance of redox homeostasis and mitochondrial function might contribute to the increased radioresistance of lung cancer cells with tolerance to chronic hypoxia/reoxygenation stress. However, the role of SLC25A1 for the cellular radiation response has not yet been investigated. Therefore, in the present study we aimed to explore the role of SLC25A1 for the increased antioxidant capacity of cancer cells adapted to chronic cycling severe hypoxia/reoxygenation stress and the use of SLC25A1 inhibition as novel strategy for radiosensitization of NCI-H460 lung adenocarcinoma cells exposed to acute or chronic cycling severe hypoxia.

RESULTS

Acute and Chronic-Cycling Hypoxia Increase Expression of *SLC25A1* and *IDH2*

To gain insight into a potential relevance of SLC25A1 for tolerance of lung cancer cells to chronic cycling severe hypoxia, we used our established cell model of so-called "anoxia-tolerant" NCI-H460 lung cancer cells and the respective control cells termed "oxic" NCI-H460 cells. These cells had been exposed to 25 cycles of severe hypoxia (48 h) and reoxygenation stress (120 h) as described earlier (24). Quantitative real-time PCR (qRT-PCR) analysis revealed a significant upregulation of *SLC25A1* in the anoxia-tolerant NCI-H460 cells as compared with the oxic control cells under standard culturing conditions, suggesting that basal upregulation of *SLC25A1* might be a consequence of adaptation to chronic cycling severe hypoxia (**Figure 1A**). *SLC25A1* upregulation was associated with upregulation of *IDH2*, whereas *IDH1* expression was not altered (**Figure 1A**). To test a more general relevance of these findings, we additionally examined the expression of the respective genes in similarly generated anoxia-tolerant DU145 and T98G cells and again observed an upregulated basal *SLC25A1* and *IDH2* expression in the anoxia-tolerant cells as compared to the respective oxic control cells (**Figures 1B,C**), whereas *IDH1* expression was not altered. Interestingly, exposure to acute severe hypoxia (0.2% O_2) was also able to trigger increased expression of *SLC25A1* and *IDH2* in the lung cancer cells and this effect was observed in both, oxic and anoxia-tolerant NCI-H460 cancer cells, compared to the oxic NCI-H460 control cells under normoxic (Nx) conditions (**Figures 1D,E**). However, the apparent upregulation of *SLC25A1* and *IDH2* expression induced by acute hypoxia was not significant for anoxia-tolerant NCI-H460 cells as the major increase over the levels of oxic NCI-H460 control cells in normoxia was already caused by the adaptation to chronic cycling severe hypoxia, whereas exposure to acute hypoxia had only a minor addition effect (Figures S1D,E in Supplementary Material). Similar observations about a significant upregulation of SLC25A1 expression upon exposure of NCI-H460 cells to acute or chronic cycling severe hypoxia were made using Western blot analysis (Figures S1A,B in Supplementary Material).

Overexpression of SLC25A1 in Lung Cancer Is Associated With Reduced Overall Survival of Lung Cancer Patients

To investigate whether upregulation of SLC25A1 in an adverse microenvironment *in vitro* might be relevant for the clinical situation, we searched for and analyzed the data of Kaplan–Meier plotter tool (kmplot.com) (40–42), about SLC25A1 expression in lung cancer patients (**Figures 2A,C**) and normal lung tissue by an *in silico* analysis, respectively (**Figure 2B**). The patient cohort has been described in detail in Ref. (40), whereas the parameters used for our *in silico* analysis are given in Tables S1 and S2 in Supplementary Material. We split the lung cancer patient cohort by median of SLC25A1 expression into "High" and "Low," respectively. Our *in silico* analysis revealed that SLC25A1 overexpression was associated with significantly reduced overall survival and median survival in lung cancer patients. Interestingly, this effect regarding overall and median survival was enhanced in the cohort of patients with successful surgery with tumor-free margins (R0-resection) (**Figure 2C**). Of note, SLC25A1 displayed a higher expression in lung cancer patients compared to normal lung tissue (**Figure 2B**) suggesting that SLC25A1 might be a relevant target in lung cancer.

Pharmacologic Inhibition of SLC25A1 Sensitizes Cancer Cells to the Cytotoxic Action of Ionizing Radiation (IR) and Overcomes Increased Radioresistance Induced by Chronic Cycling Severe Hypoxia

So far, our data demonstrated that acute and chronic cycling severe hypoxia alter the expression levels of SLC25A1. Moreover, our earlier data revealed that anoxia-tolerant NCI-H460 cells are more resistant to IR and chemotherapeutic agents compared to non-selected oxic control cells and this increased radioresistance could be linked to improved antioxidant defense of anoxia-tolerant cancer cells (22–24). Since SLC25A1 has recently been linked to cellular redox homeostasis (36) we wondered whether SLC25A1 might contribute to increased radioresistance of cancer cells in acute or chronic cycling severe hypoxia. To test a potential influence of SLC25A1 on radiosensitivity, we treated the anoxia-tolerant NCI-H460 cells and the oxic control cells with the pharmacological SLC25A1-inhibitor 1,2,3-benzene-tricarboxylic acid (BTA) (38, 39) 2 h before irradiation with 0–5 Gy under standard Nx conditions (20% O_2) and determined the effects of single and combined treatment on cell survival in standard long-term colony survival assays upon removal of BTA 24 h after treatment (delayed plating) (experimental timeline, **Figure 3A**). To test the impact of drug-treatment in acute hypoxia, we additionally performed similar experiments in cells that had been adapted to acute severe hypoxia (0.2% O_2) for 2 h prior to inhibitor treatment (**Figure 3A**).

Our results revealed that treatment with the SLC25A1-inhibitor BTA sensitized both oxic and anoxia-tolerant NCI-H460 cancer cells to the cytotoxic action of IR when treatment was performed under Nx conditions (**Figures 3B,D**). As expected, the effect was more pronounced in the anoxia-tolerant cells with increased

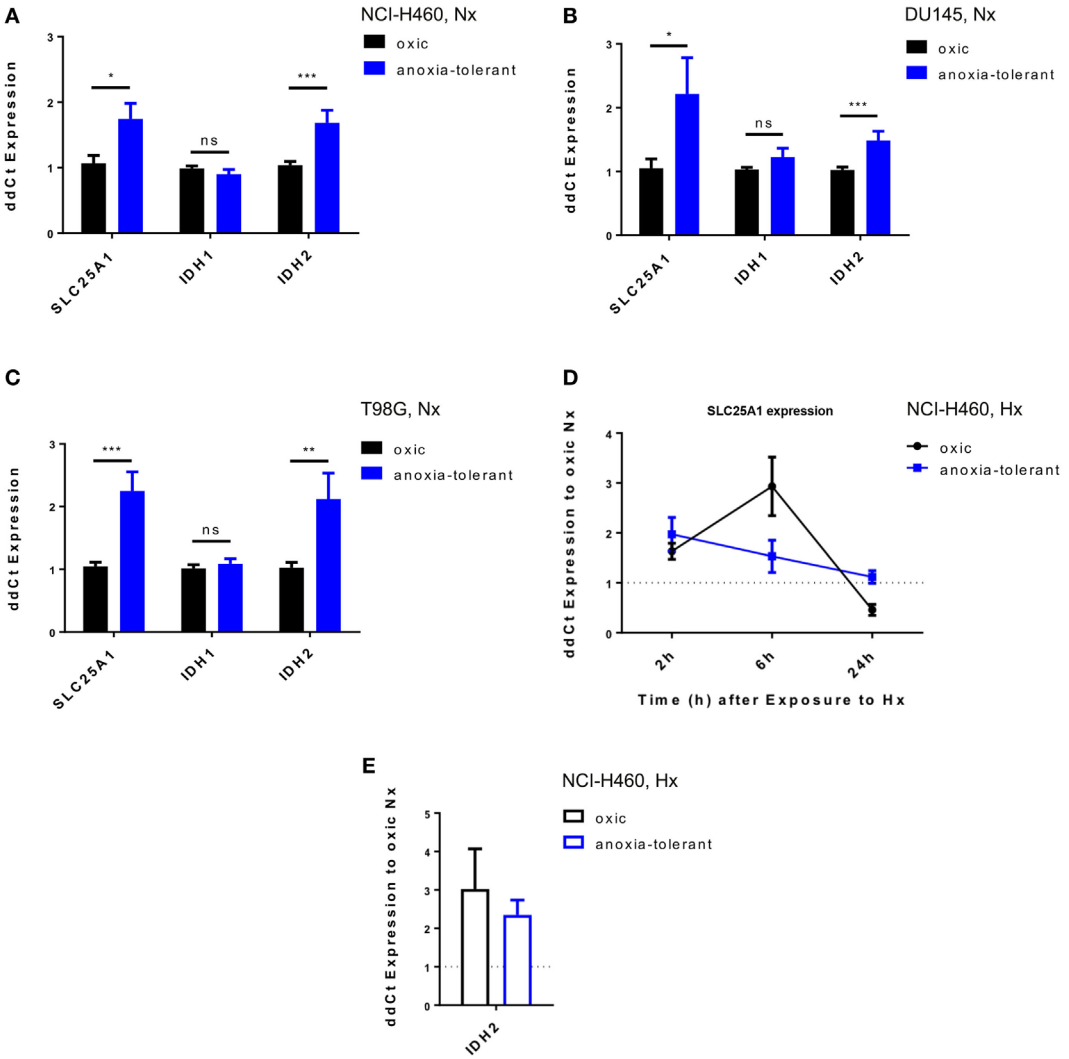


FIGURE 1 | Exposure of cancer cells to acute or chronic cycling severe hypoxia leads to upregulated expression of *SLC25A1* and *IDH2*. Expression of *SLC25A1*, isocitrate dehydrogenase 1 (*IDH1*; cytosolic), and isocitrate dehydrogenase 2 (*IDH2*; mitochondrial) was determined in anoxia-tolerant cancer cells generated by exposure to 16 cycles (T98G) or 25 cycles (NCI-H460, DU145) of severe hypoxia (48 h, <0.1% O_2) and reoxygenation (120 h air plus 5% CO_2 referred as 20% O_2) and the respective oxic control cells (24) by quantitative real-time PCR analysis. Data show basal expression of *SLC25A1*, *IDH1*, and *IDH2* in **(A)** NCI-H460, **(B)** DU145, and **(C)** T98G oxic and anoxia-tolerant cells under normoxic (Nx) conditions (20% O_2). Changes in *SLC25A1* [2–24 h, **(D)**] and *IDH2* [24 h, **(E)**] expression in oxic and anoxia-tolerant NCI-H460 cells in response to acute severe hypoxia (Hx, 0.2% O_2). Data were always normalized to the respective oxic control cells under basal Nx conditions. Mean values ± SEM are shown, $n = 3$ (*$p \leq 0.05$, **$p < 0.01$, and ***$p \leq 0.001$; *t*-test).

SLC25A1 expression and higher radioresistance. Interestingly, radiosensitization was also observed when treatment was performed in acute severe hypoxia (**Figures 3C,E**). BTA treatment was even able to partially compensate the reduced efficacy of IR in acute hypoxia (**Figure 3E**). Remarkably, we observed these effects even though BTA was already removed 24 h after IR so that long-term incubation was performed in inhibitor-free media. To confirm the relevance of SLC25A1 inhibition for the radiorsensitizing effects of BTA, we performed additional experiments with a chemically distinct SLC25A1-inhibitor 4-Chloro-3-[[(3-nitrophenyl)amino]sulfonyl]-benzoic acid (CNASB), with higher specificity for SLC25A1 (43). These experiments revealed that combined treatment of CNASB and IR similarly sensitized both, oxic and anoxia-tolerant NCI-H460 cancer cells, to the cytotoxic action of IR under Nx and hypoxic (Hx) conditions in short-term proliferation (Figure S4A in Supplementary Material) and long-term survival assays (Figure S4C in Supplementary Material) corroborating the radisensitizing action of BTA at the level of SLC25A1.

Cellular and Mitochondrial Redox Homeostasis Is Impaired After Inhibition of SLC25A1

As demonstrated in NCI-H460 cells IDH1, IDH2, and SCL25A1 are all part of a pathway responsible for the bidirectional

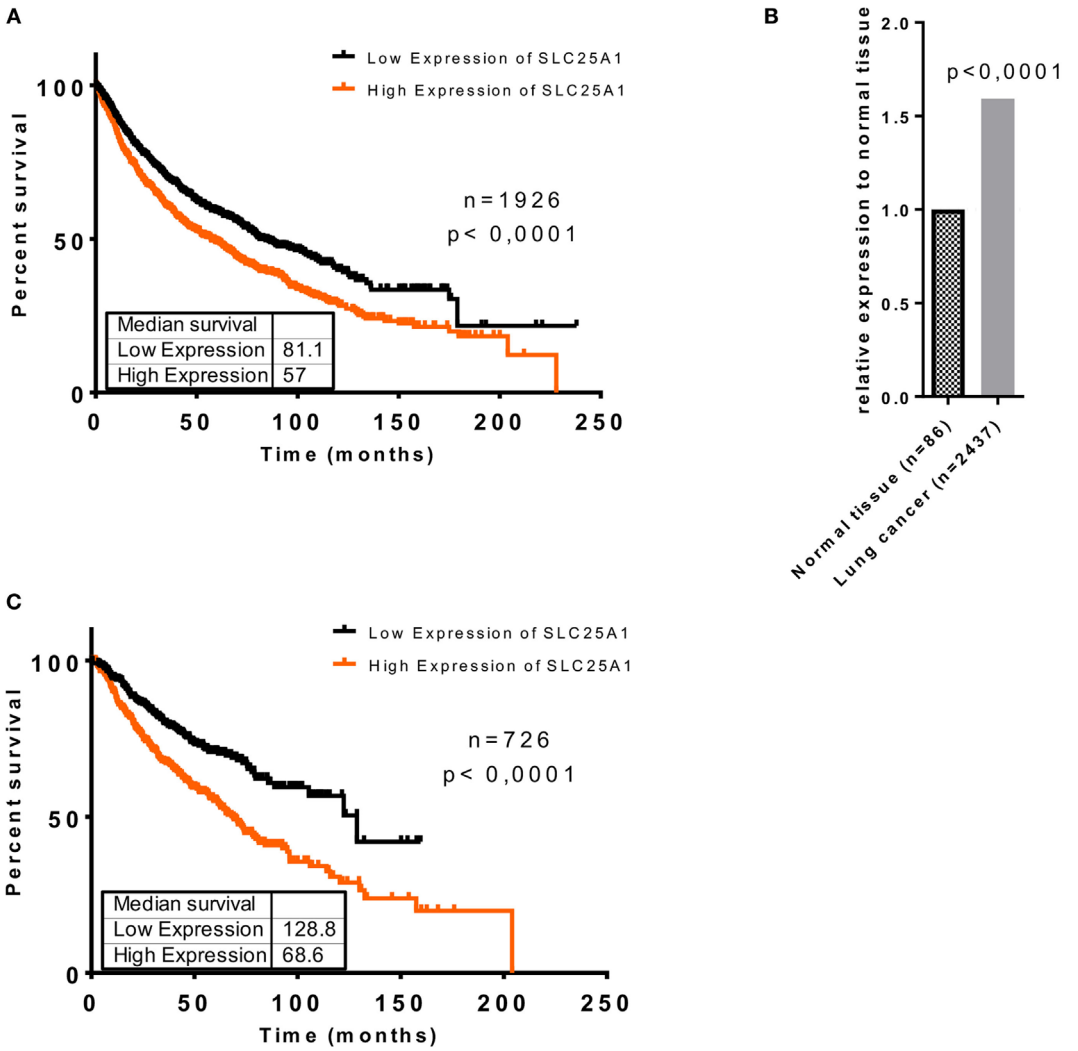


FIGURE 2 | Elevated expression of SLC25A1 is associated with worse clinical outcome of lung cancer patients, particularly upon tumor resection with negative margins (R0). Clinical relevance of the SLC25A1 expression was obtained by searching and analyzing available patient array data by the Kaplan–Meier plotter tool (kmplot.com). Data show **(A)** overall survival in lung cancer patients, **(B)** relative expression of SLC25A1 in normal tissue and lung cancer, and **(C)** overall survival in lung cancer patients upon surgery with negative tumor margins (R0). For the analysis, the cohort was split by median of SLC25A1 expression ("High" and "Low," respectively). Median survival (in months) is indicated for each survival curve.

transport of NADPH over the mitochondrial inner membrane (36). We therefore investigated whether the cytotoxic and radiosensitizing effects of the acute BTA treatment might be linked to alteration of cellular redox homeostasis. To this end, we measured total NADPH level (NADPH + $NADP^+$) by using a luciferase-coupled enzymatic reaction. As shown in **Figure 4A,** anoxia-tolerant NCI-H460 cells were characterized by a significant increase of total NADPH, presumably as a consequence of adaptation to chronic cycling severe hypoxia, whereas treatment with BTA for 2 h reduced the total NADPH level to the levels of the untreated oxic control cells (**Figure 4A**). In contrast, BTA had no significant effect on total NADPH levels in the oxic control cells (**Figure 4A**). We observed less pronounced effects regarding the BTA-mediated depletion of total NADPH levels in acute hypoxia (**Figure 4B**). Interestingly, the $NADP^+$/NADPH ratios were slightly increased after BTA treatment especially in anoxia-tolerant NCI-H460 cancer cells compared to respective controls in normoxia and hypoxia (**Figures 4C,D**).

NADPH is required amongst others for the regeneration of the reduced form of glutathione (GSH). We therefore next examined the effect of BTA treatment on cellular glutathione levels (**Figures 4E,F**). In line with our previous findings (24), anoxia-tolerant NCI-H460 cancer cells had elevated GSH levels compared to oxic control cells (**Figure 4E**). As expected, BTA treatment significantly reduced GSH levels in oxic and anoxia-tolerant NCI-H460 cancer cells when BTA treatment was performed under Nx conditions (**Figure 4E**). Similar observations were made when cells were treated with BTA in acute severe hypoxia (**Figure 4F**). Of note, BTA decreased the GSH levels of

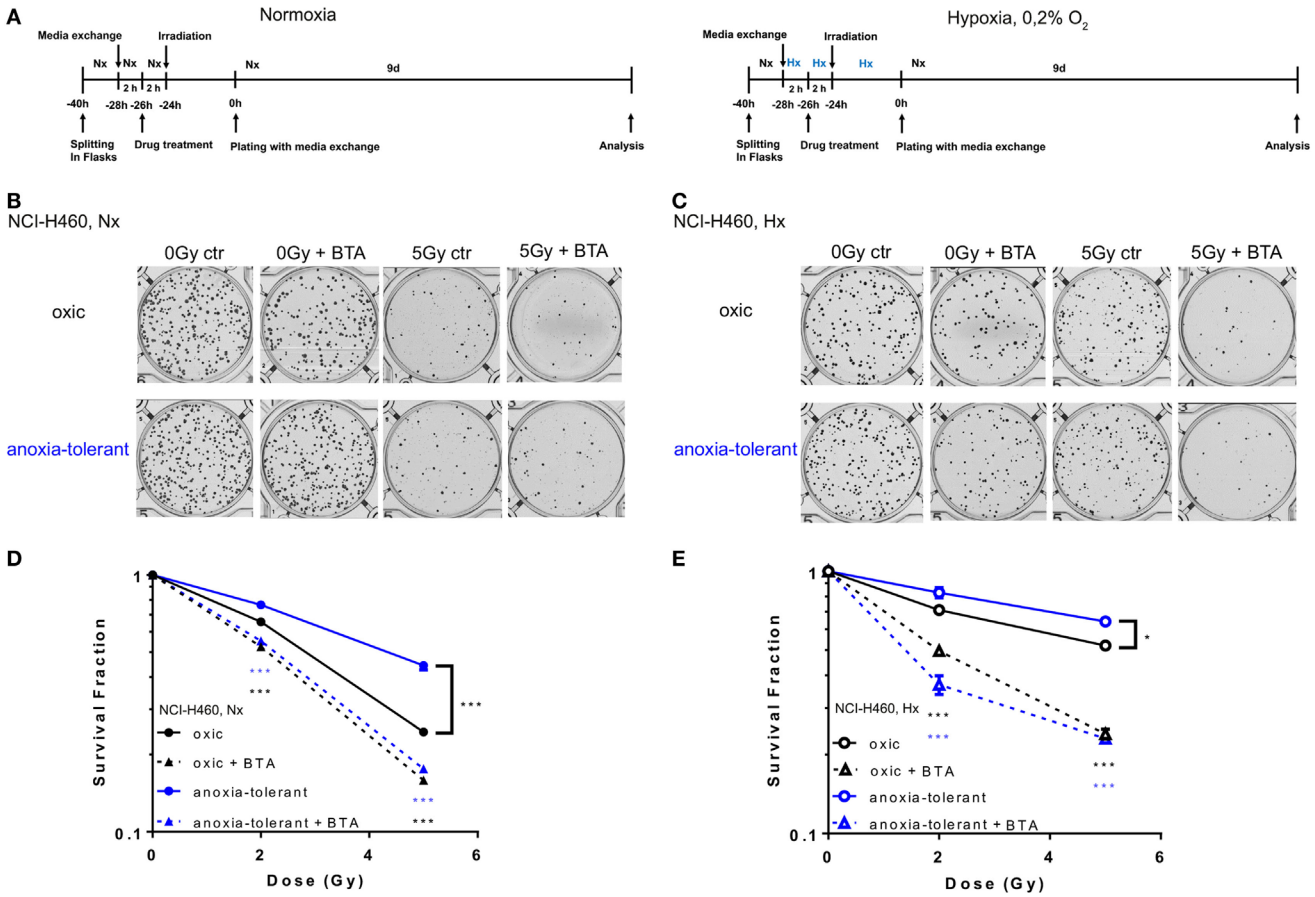


FIGURE 3 | Pharmacologic inhibition of SLC25A1 overcomes chronic cycling hypoxia-induced radioresistance. Anoxia-tolerant NCI-H460 cells and the oxic NCI-H460 control cells were pretreated with 5-mM BTA (2 h prior to IR) under normoxic (Nx) conditions (20% O_2) or upon 2-h preincubation in severe hypoxia (Hx, 0.2% O_2). Twenty-four hours after treatment cells were collected, plated at different cell numbers (200–3,200) in full medium without the inhibitor and grown under Nx for 9 days. **(A)** Schematic representation of the experimental timeline in normoxia (Nx, left panel) or severe Hx (right panel). Photomicrographs in **(B)** and **(C)** show representative pictures of colony formation upon treatment with IR without or with prior BTA treatment for Nx **(B)** or severely hypoxic conditions **(C)**. Survival curves shown in **(D,E)** depict quantification of colony formation upon treatment in normoxia **(D)** and severe hypoxia **(E)**. Colonies were scanned and counted using GelCount. Survival fractions were calculated to the plating efficiency of unirradiated cells under Nx or Hx conditions. Mean values ± SEM are shown, $n = 3$ (*$p \leq 0.05$, **$p < 0.01$, and ***$p \leq 0.001$; two-way ANOVA with Tukey post-test.) Comparisons between treatment and corresponding controls at the same dose are indicated.

the anoxia-tolerant cancer cells to the levels of the oxic control cells (**Figures 4E,F**).

To further specify the impact of the SLC25A1-inhibitor BTA on cellular antioxidant capacity, particularly mitochondrial and cellular redox homeostasis, we measured mitochondrial and cellular ROS after BTA treatment in normoxia and acute severe hypoxia (**Figures 4G,H**; Figure S2 in Supplementary Material). Importantly, BTA treatment increased the levels of mitochondrial ROS in oxic and anoxia-tolerant NCI-H460 cancer cells under Nx conditions (**Figure 4G**). This effect was even enhanced when BTA treatment was performed in acute hypoxia (**Figure 4H**; Figure S2 in Supplementary Material). Additionally, cellular ROS-levels were increased in both, oxic and anoxia-tolerant cells, upon BTA treatment in normoxia and acute severe hypoxia (Figures S2E,F in Supplementary Material). Nevertheless, BTA-induced cellular ROS levels were lower compared to the mitochondrial ROS levels (Figures S2 in Supplementary Material).

Taken together, the SLC25A1-inhibitor BTA efficiently decreased cellular NADPH and GSH levels resulting in increased mitochondrial and cellular ROS. These findings point to a role of SLC25A1 in the regulation of cellular antioxidant capacity and mitochondrial redox homeostasis.

SLC25A1 Inhibition Lowers Mitochondrial Metabolism and Alters Metabolic Demand

So far, our data indicated that increased production of mitochondrial ROS upon SLC25A1 inhibition by BTA treatment might play a role in BTA-mediated radiosensitization. However, SLC25A1 is of broader relevance for the transport of metabolites between mitochondrial intermembrane space and mitochondrial matrix as it shuttles isocitrate, malate and phosphoenolpyruvate, in addition to citrate (38, 44). We therefore wondered whether BTA

might have a more general effect on mitochondrial metabolism and therefore additionally measured the effects of BTA on mitochondrial metabolism by using extracellular flux measurements (**Figure 5**).

Pretreatment of cells with BTA for 24 h led to decreased basal mitochondrial respiration and lowered ATP-Production in both, oxic and anoxia-tolerant NCI-H460 cells, as determined by using the Mito Stress Test (**Figure 5A**). Additionally, we observed a decreased ability of cells treated with the SLC25A1 inhibitor to respond to forced mitochondrial respiration by uncoupling the electronic transport chain, an effect that is termed reduced spare respiratory capacity (**Figure 5A**, left panel). As shown in **Figure 5A,** the inhibitory effect was more pronounced in the anoxia-tolerant NCI-H460 cells as these cells were found to dispose of an increased spare respiratory capacity (**Figure 5A**, right panel) in comparison to oxic control cells (**Figure 5A**, middle

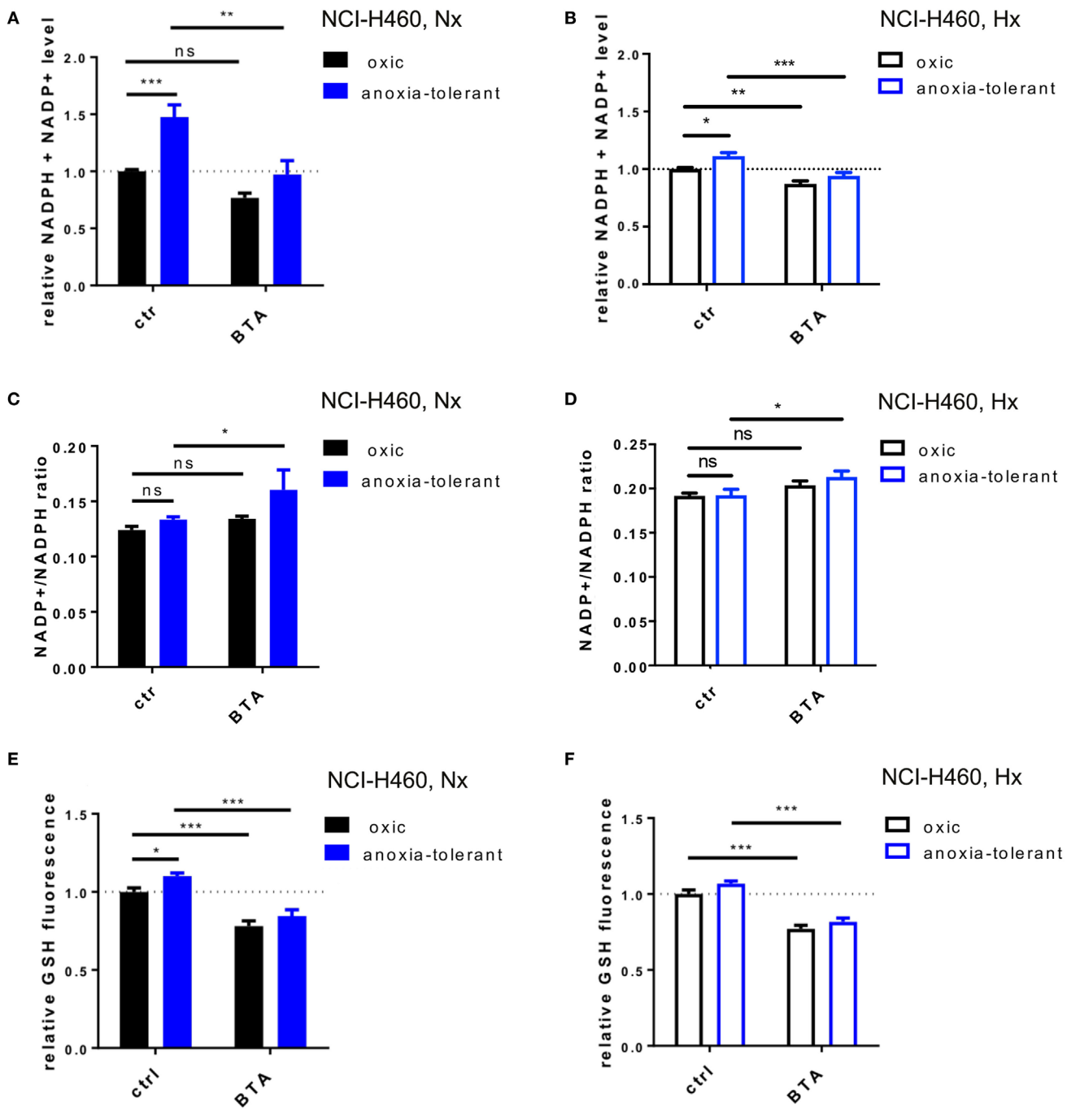


FIGURE 4 | Continued

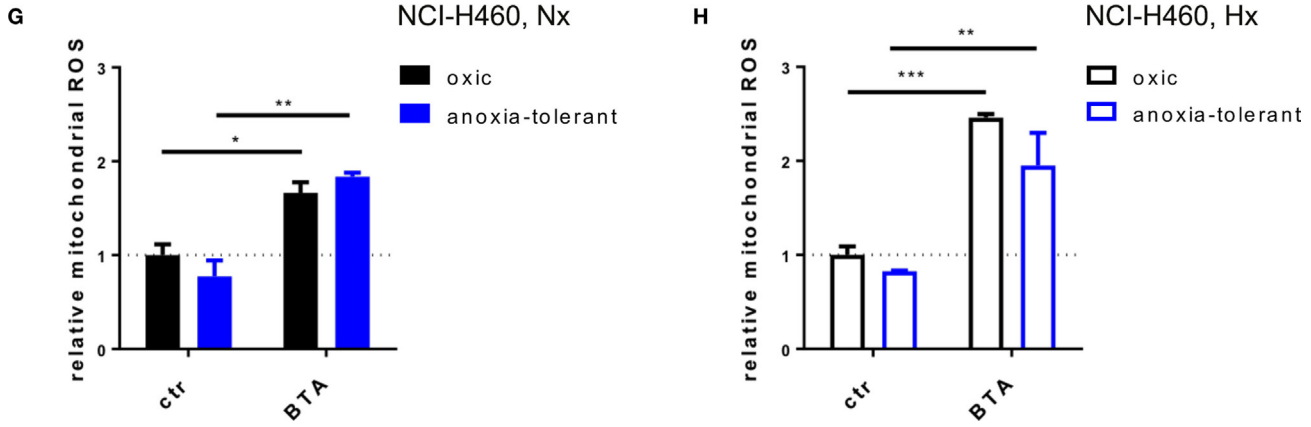


FIGURE 4 | Inhibition of SLC25A1 disturbs cellular and mitochondrial redox homeostasis. NCI-H460 oxic and anoxia-tolerant cells were treated with 5 mM BTA in normoxia (Nx, 20% O_2) or upon preincubation for 2 h in acute severe hypoxia (Hx, 0.2% O_2) and antioxidant capacity and ROS formation were determined 2 or 6 h after treatment in functional assays. **(A)** Impact of BTA treatment on total NADPH levels (NADPH + $NADP^+$) under normoxic (Nx) conditions as determined by using a luciferase-coupled enzymatic reaction 2 h after treatment. **(B)** Impact of BTA treatment on total NADPH levels (NADPH + $NADP^+$) under Hx conditions. **(C)** $NADP^+$/NADPH ratio upon 2 h of BTA treatment under Nx conditions. **(D)** $NADP^+$/NADPH ratio upon 2 h of BTA treatment under Hx conditions. **(E)** Influence of BTA treatment on GSH levels under Nx conditions as measured by fluorescence of monochlorobimane (MCB) 2 h after treatment. **(F)** Influence of BTA treatment on GSH levels under Hx conditions. **(G)** Mitochondrial superoxide reactive oxygen species (ROS) as determined in flow cytometry by staining with MitoSox 6 h after treatment under Nx conditions. Fold changes of gated positive cells are indicated. **(H)** Mitochondrial ROS as determined in flow cytometry by staining with MitoSox 6 h after treatment under Hx conditions. Mean values ± SEM are shown, $n = 3$. (ns $p > 0.05$, $^*p \leq 0.05$, $^{**}p < 0.01$, and $^{***}p \leq 0.001$; two-way ANOVA with Tukey post-test.)

panel). Similar effects on cell metabolism were observed when using CNASB, another more potent small molecule inhibitor of SLC25A1 (Figure S4B in Supplementary Material).

But inhibition of SLC25A1 might also impact the metabolic demands of oxic and anoxia-tolerant NCI-H460 cells to maintain mitochondrial function. To identify the metabolic pathways needed to maintain mitochondrial respiration under BTA treatment we performed the Fuel Flex Test. In this test the glucose/pyruvate pathway was inhibited by UK5099, the glutamine pathway by using BPTES and the fatty acid (FA) pathway by etomoxir treatment, respectively. Overall, BTA treatment induced a higher dependency of oxic and anoxia-tolerant NCI-H460 cells on the final glycolytic product pyruvate as well as glutamine to maintain mitochondrial respiration (**Figure 5B**). Furthermore, combined inhibition of two of these pathways reduced the capacity to maintain mitochondrial respiration only for glucose/pyruvate and FAs but not for glutamine when the other two pathways were inhibited (**Figure 5B**). This suggests that BTA reduces the capacity and flexibility of oxic and anoxia-tolerant cells to oxidize other fuels, when the pathway of interest is inhibited. Since BTA treatment lowered the flexibility of oxic and anoxia-tolerant cell to oxidize FAs, when glucose/pyruvate or glutamine pathways were inhibited, we measured FA uptake upon BTA treatment. We observed a reduction in the uptake of labeled FAs following BTA treatment (**Figure 5C**). Taken together, BTA treatment lowered the metabolic flexibility for all metabolic pathways examined in both oxic and anoxia-tolerant NCI-H460 cells, as defined by the difference between metabolic dependency and capacity (**Figure 5B**) and lead to reduced FA uptake (**Figure 5C**).

These findings revealed a role of SLC25A1 in conserving mitochondrial metabolism. Importantly, the enhanced flexibility of mitochondrial metabolism as a consequence of upregulated SLC25A1 expression might contribute to increased stress-tolerance of the NCI-H460 cancer cells.

Inhibition of SLC25A1 Affects the Repair of IR-Induced Double-Strand Breaks (DSB)

So far, our data indicated that pharmacologic inhibition of SLC25A1 by BTA or CNASB increases radiosensitivity of oxic and anoxia-tolerant NCI-H460 cancer cells and that disturbance of the redox homeostasis and of metabolic flexibility might participate in the radiosensitizing effects. Herein, one important aspect of radiosensitivity is the ability of the cells to repair IR-induced DNA DSB. Therefore, we finally examined if BTA treatment would affect the time-dependent induction and resolution of DNA-repair foci positive for Histone H2A.X phosphorylated at serine 139 (γH2AX), a marker for DNA DSB (45). BTA treatment alone (without IR) did not induce additional DSB, ruling out a direct effect of BTA on DNA-damage induction and repair (**Figures 6A,B**). Interestingly, the presence of BTA during exposure to IR slowed the resolution of IR-induced γH2AX-foci and led to higher residual amount of DNA lesions 24 h after IR-treatment in Nx (**Figures 6A,C**) and even in Hx conditions (**Figures 6B,D**). Surprisingly, despite the differences in SLC25A1 expression between oxic and anoxia-tolerant NCI-H460 cells, BTA treatment had similar effects on DSB repair in both cell lines. Even more important, despite the obvious differences in radiosensitivity, the kinetics of DSB repair as determined by the resolution of IR-induced γH2AX-foci were rather similar in oxic and anoxia-tolerant NCI-H460 cells. This suggests that other parameters of the DNA damage response might dictate the differences in radiosensitivity, presumably the differences in

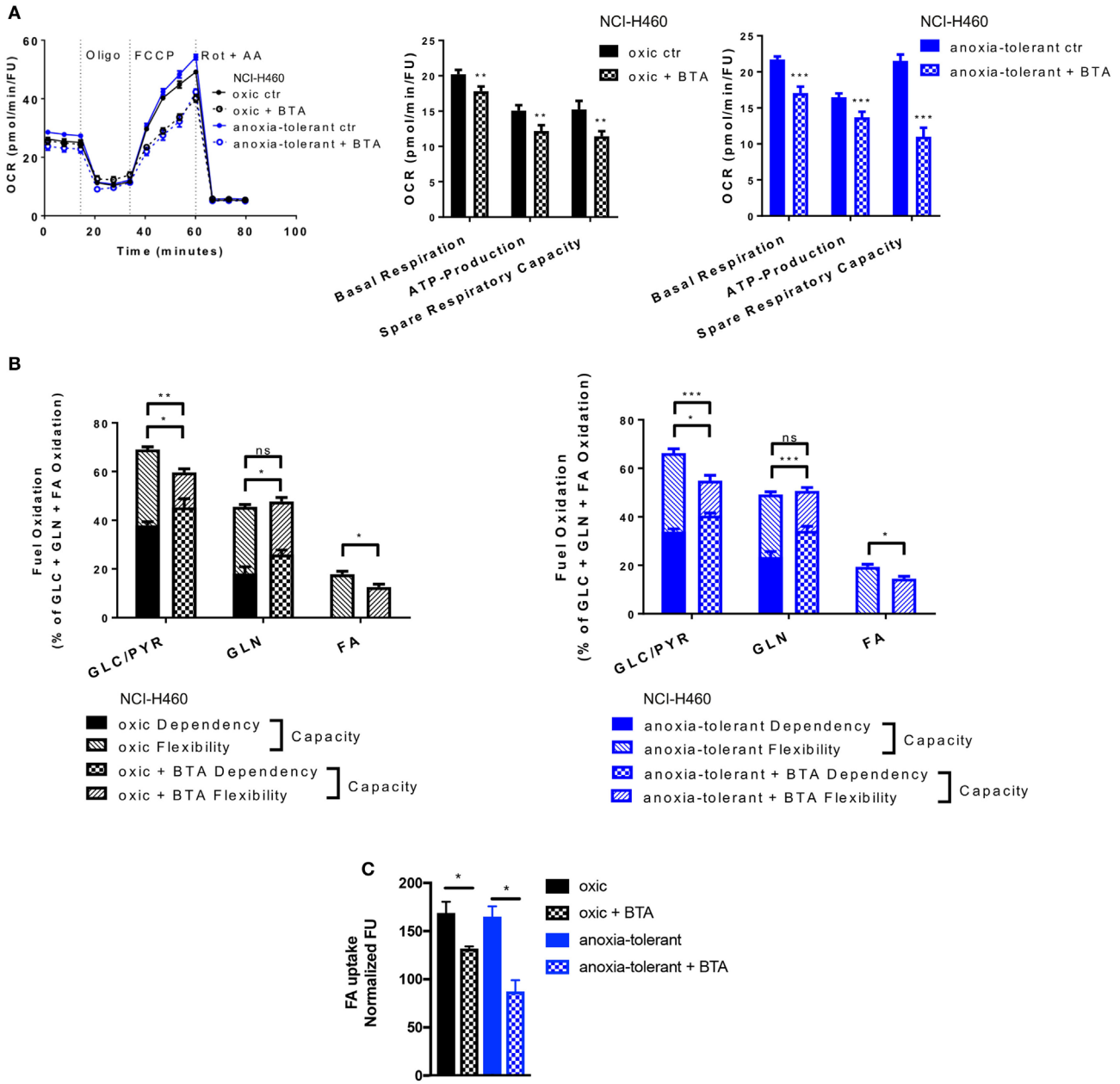


FIGURE 5 | Metabolic alterations upon SLC25A1 Inhibition in NCI-H460 oxic and anoxia-tolerant cells. NCI-H460 oxic and anoxia-tolerant cells were treated for 24 h with 5 mM 1,2,3-benzene-tricarboxylic acid (BTA) under standard ambient conditions and various parameters of cell metabolism were determined by using a Seahorse XFe 96 analyzer. Fatty-acid (FA) uptake was measured by using the fluorescent FA analog C1-BODIPY® 500/510 C12. **(A)** Oxygen consumption rate (OCR) was measured using Mito Stress Test Kit and normalized to Hoechst 33342 fluorescence units (FU) 24 h after treatment with BTA. Real-time Injection of oligomycin (Oligo, 1 μM), carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (FCCP, 2 μM), Rotenone (Rot, 0.5 μM) and antimycin A (AA, 0.5 μM). Calculated parameters of the assay are indicated in bar graphs. **(B)** Percentage of total mitochondrial respiration for each fuel pathway measured with Seahorse XFe 96 analyzer using Fuel Flex Test Kit 24 h after treatment with BTA (fuel pathways: GLC/PYR: Glucose/Pyruvate, inhibition by UK5099; GLN, glutamine, inhibition by BPTES, FA: fatty acids, inhibition by etomoxir). **(C)** FA uptake measured after 24 h of BTA treatment. Mean values ± SEM are shown. Data in **(A)** and **(B)** from $n = 12–18$ wells from two independent experiments; Data in **(C)** $n = 3$ (*$p \leq 0.05$, **$p < 0.01$, and ***$p \leq 0.001$. **(A)** t-test; **(B)** and **(C)** two-way ANOVA with Tukey post-test).

the ability to cope with radiation-induced ROS. Nevertheless, it was an interesting observation that BTA was able to retard the resolution of IR-induced γH2AX-foci and cause a higher amount of residual DNA lesions 24 h after irradiation.

Interestingly, it had recently been shown that a knockout of SLC25A1 not only impairs mitochondrial function, resulting in higher glycolysis rate and the usage of glutamine for compensation of impaired FA synthesis, but also leads to the accumulation of

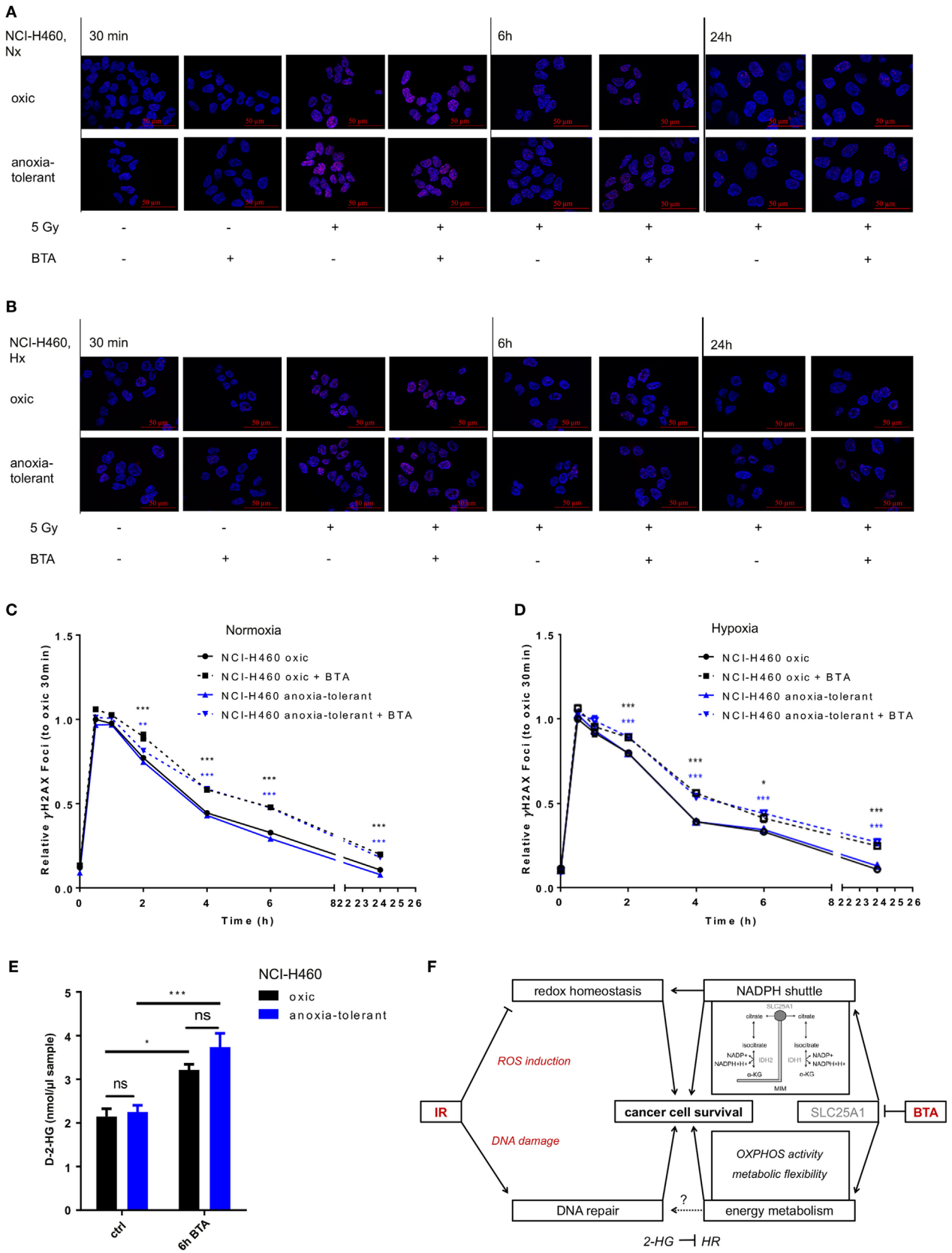


FIGURE 6 | Continued

FIGURE 6 | Inhibition of SLC25A1 affects the repair of radiation-induced DNA damage. NCI-H460 oxic and anoxia-tolerant cells were left untreated or exposed to ionizing radiation (IR) with a single dose of 5 Gy with or without prior preincubation of the cells for 2 h with 5 mM 1,2,3-benzene-tricarboxylic acid (BTA) under normoxic (Nx, 20% O_2) and hypoxic (Hx, 0,2% O_2) conditions. **(A)** Time-dependent accumulation of γH2AX foci in irradiated NCI-H460 oxic and anoxia-tolerant cells under Nx conditions was evaluated by fluorescence microscopy without and with additional treatment with 5 mM BTA at indicated time points after IR. **(B)** Time-dependent accumulation of γH2AX foci in irradiated NCI-H460 oxic and anoxia-tolerant cells under Hx conditions was evaluated by fluorescence microscopy without and with additional treatment with 5-mM BTA at indicated time points after IR. **(C)** Mean number of γH2AX foci per cell after IR without and with additional BTA treatment in Nx was calculated with the Focinator v2 software and normalized to oxic control cells 30 min after IR. **(D)** Mean number of γH2AX foci per cell after IR without and with additional BTA treatment in Hx was calculated with the Focinator v2 software and normalized to oxic control cells 30 min after IR. **(E)** D-2-hydroxyglutarate levels (D-2-HG) were determined in lysed cells 6 h after treatment with BTA in Nx. **(F)** Schematic representation of the suggested mechanisms of the actions of BTA and IR on cancer cell survival. Mean values ± SEM are shown, $n = 3$ (*$p \leq 0.05$, **$p < 0.01$, and ***$p \leq 0.001$; two-way ANOVA with Tukey post-test. Comparisons between treatment and corresponding controls at the same time point are indicated). MIM, mitochondrial inner membrane; α-KG, α-Ketoglutarate; HR, homologous recombination.

2-hyodroxyglutarate (2-HG) in NCI-H460 cells (44). Of note, accumulation of the oncometabolite 2-HG has been linked to DNA-repair defects (46). We therefore wondered whether BTA-mediated inhibition of SLC25A1 might have similar effects and measured the effects of BTA treatment on the level of D-2-HG. For this, we used an enzymatic reaction detecting a colored product photometrically at 450 nm. As shown in **Figure 6E**, BTA treatment indeed significantly increased the levels of D-2-Hydroxyglutarate (D-2-HG) 6 h after BTA treatment in both oxic and anoxia-tolerant NCI-H460 cells (**Figure 6E**). This increase in 2-HG might thus be responsible for the observed delay in the kinetics of the repair of radiation-induced DNA DSB observed in both cell lines (**Figures 6A–D**). The finding that there was no significant difference in the absolute D-2-HG levels upon BTA treatment between oxic control and anoxia-tolerant NCI-H460 might explain why the BTA-induced retardation in DNA repair was similar in both cell lines. Taken together our novel findings suggest that BTA exerts a dual effect on the cellular radiation response by targeting both DNA repair and metabolic escape mechanisms of aggressive cancers making it a promising radiosensitizer.

DISCUSSION

Tumor hypoxia is an important biological factor causing poor therapy outcome and worse prognosis in NSCLC patients. However, so far hypoxia-targeting strategies have not been translated into clinical practice highlighting the need for an improved understanding of the underlying mechanisms if we aim to develop more effective strategies for therapeutic intervention. In our work, we focus on mechanisms of radiation resistance caused by adaptation of cancer to an adverse Hx environment.

Our previous work identified enhanced antioxidant capacity based on glutamine-dependent glutathione-regeneration as a mechanism favoring stress tolerance and radioresistance of cancer cells with tolerance to chronic cycling severe hypoxia (23, 24). Here, we reveal important novel mechanistic aspects of increased antioxidant capacity in cancer cells and demonstrate a therapeutic potential for SLC25A1 inhibition to overcome radioresistance in chronically Hx lung cancer cells: (i) Exposure of cancer cells to acute or chronic cycling severe hypoxia was associated with upregulated expression of the citrate transporter SLC25A1. (ii) Pharmacologic inhibition of SLC25A1 by BTA reduced cellular antioxidant capacity, enhanced the generation of mitochondrial ROS and abrogated the increase in radioresistance of lung cancer cells induced by adaptation to chronic cycling severe hypoxia. (iii) BTA treatment was also associated with a pronounced disturbance of mitochondrial metabolism, accumulation of the oncometabolite 2-HG, and impaired repair of radiation-induced DNA DSB. These findings point to a yet unknown role of SLC25A1 in radioresistance that might be linked to regeneration of GSH for ROS-detoxification and metabolic regulation of DNA repair. Remarkably, we confirmed the metabolic and radiosensitizing effects of SLC25A1-inhibition also with CNASB, another potent small-molecule inhibitor with documented higher specificity to SLC25A1 (43). In line with our observations using BTA the radiosensitizing effects of CNASB were more prominent in the anoxia-tolerant cancer cells. This further corroborates our assumption that chronic cycling hypoxia/reoxygenation stress increases not only the expression but also the reliance of cancer cells on the SLC25A1 mediated redox-homeostasis for survival under stress conditions.

Interestingly, high SLC25A1 expression in lung cancer patients has already been linked with poorer overall survival compared to lung cancer patients with low SLC25A1 expression in an earlier report (42). However, here we expand these findings with respect to the importance of SLC25A1 in terms of recurrence and progression of lung cancer suggesting that SLC25A1 may be an attractive therapeutic target for tumor cell-specific radiosensitization at least in patients with high SLC25A1 expression. Of note, mutant p53 can promote transcriptional activation of SLC25A1 eventually by an interaction with the FOXO-1 transcription factor (42). This suggests that SLC25A1 might mediate some of the oncogenic activities of mutant p53—an important prognostic marker predictive for relapse and resistance to chemotherapy and RT (47).

In addition, upregulated expression of SLC25A1 was always associated with upregulation of IDH2. Thus, SLC25A1 and IDH2 might cooperate in adaptive metabolic processes that allow cancer cells to cope with the adverse conditions in the tumor microenvironment. Others have described a crucial role of the presence of active IDH2 for proliferation and survival of glioblastoma cancer cells in hypoxia (48).

However, our primary goal was to evaluate the use of SLC25A1 as a therapeutic target to overcome radioresistance in chronically Hx lung cancer cells. It has been shown earlier that inhibition of SLC25A1 by BTA reduces the growth of xenograft tumors

of breast, bladder or lung cancer cells without any evidence for additional normal tissue toxicity (39). But here we demonstrate for the first time that SLC25A1 has a role in lung cancer cell radiation resistance. For this we measured the effects of the SLC25A1-inhibitor BTA on radiation-induced eradication of clonogenic tumor cells in long-term colony formation assays (delayed plating) upon treatment in combination with IR. Our results revealed that treatment with BTA for 24 h was sufficient to significantly reduce the survival fraction of both, anoxia-tolerant NCI-H460 cells and the respective oxic NCI-H460 control cells. However, the radiosensitizing effect was more pronounced in the anoxia-tolerant NCI-H460 cells and could be further enhanced by treatment in acute severe hypoxia. Of note, we made similar observations when using a chemically distinct small molecule SLC25A1 inhibitor, CNASB, thereby corroborating the action of BTA at the level of SLC25A1. Moreover, the latter finding underlines the importance of SLC25A1-controlled metabolic and redox related alterations for adaptation to acute and chronic cycling hypoxia.

Mechanistically, we discovered that BTA exerts a dual effect on the cellular radiation response by targeting both, cell metabolism and DNA repair, making it a promising radiosensitizer. On the one hand, radiosensitizing effect of the SLC25A1-inhibitor BTA was associated with a reduction of the cellular antioxidant capacity: BTA treatment significantly reduced cellular total NADPH levels and increased $NADP^+$/NADPH ratios in the anoxia-tolerant NCI-H460 cells. Moreover, BTA reduced GSH levels in both oxic and anoxia-tolerant NCI-H460 cancer cells, even in acute severe hypoxia. The reduction in these major determinants of cellular antioxidant capacity resulted in increased production of mitochondrial ROS and this effect on cellular redox-balance was even more pronounced when treatment had been performed in acute severe hypoxia. Our findings strongly suggest that SLC25A1 plays a role for maintenance of the antioxidant defense of lung cancer cells with tolerance to acute or chronic cycling severe hypoxia with potential impact on the sensitivity of the cells to ROS-induced damage. Our findings are in line with a report about a role of SLC25A1 for redox homeostasis in NCI-H460 cancer cells obtained by a stable knockout of SLC25A1 (36). Thus, our pharmacologic approach reproduces the findings obtained by the genetic approach, but shows in addition that such effects can also be obtained under conditions of acute severe hypoxia and in cells with tolerance to chronic cycling severe hypoxia. Importantly, the above-mentioned effects were neither due to direct toxic effects of BTA neither under Nx nor under Hx conditions as shown by the lack of a relevant increase in apoptosis (Figure S3 in Supplementary Material) or total cell death (not shown) upon acute treatment up to 72 h, nor to BTA-induced alterations in *SLC25A1* expression (Figure S1F in Supplementary Material).

Though SLC25A1 inhibition by BTA led to a significant induction of mitochondrial ROS compared to the respective untreated control cells, the percentage of cells stained positive for mitochondrial ROS was below 20% (Figure S2 in Supplementary Material). We therefore assume that inhibition of SLC25A1 might have effects in addition to the alterations in the redox balance that contribute to the cytotoxic effects BTA.

SLC25A1 is responsible for the bidirectional shuttling of citrate between the mitochondria and cytosol thereby supporting redox homeostasis by delivery of isocitrate to IDH2, which in turn regenerates NADPH. But SLC25A1 also impacts biosynthetic processes such as lipid biosynthesis (42) and other cellular processes. For example, it has been demonstrated that SLC25A1 and its function in citrate export to cytosol is central for cytokine-induced inflammatory signals and maintenance of NADPH redox state in macrophage activation (37, 49). Moreover, knockout of SLC25A1 has been associated with dysregulation of mitochondrial metabolism in cancer cells (44). Therefore, we further tested the effects of adaptation to chronic cycling severe hypoxia and BTA treatment on energy metabolism. Interestingly, anoxia-tolerant NCI-H460 cells displayed enhanced basal respiration and increased ability to respond to forced mitochondrial respiration (spare respiratory capacity) when compared to oxic NCI-H460 control cells. These findings demonstrate that the mitochondrial changes caused by adaptation to chronic cycling severe hypoxia are not restricted to components of the mitochondrial apoptosis signaling cascade as described earlier (22) but extend to alterations in mitochondrial oxidative metabolism. We also found that pharmacologic inhibition of SLC25A1 by BTA or CNASB significantly decreased mitochondrial respiration and ATP production. Interestingly, treatment with BTA or CNASB particularly lowered the spare respiratory capacity of anoxia-tolerant NCI-H460 cancer cells, pointing to a possible involvement of SLC25A1 in adaptation of oxidative metabolism to metabolic stress induced by chronic cycling severe hypoxia.

In addition, BTA treatment rendered NCI-H460 oxic and anoxia-tolerant cells more dependent on glycolysis-derived pyruvate and glutamine. It has been discussed that enhanced glucose uptake and a shift toward the pentose phosphate pathway (PPP) to generate more NADPH might be a strategy of cancer cells to counteract increased ROS (50). However, cancer cells use diverse strategies to increase their antioxidant capacity (cellular GSH) (31). For example, serine catabolism can participate in mitochondrial redox control under Hx conditions (51) whereas in our hands, altered glutamine usage contributed to the regeneration of glutathione and improved ROS defense of cancer cells with tolerance to cycling severe hypoxia (24). Excessive ROS-production—caused for example by oncogene-induced proliferation—causes oxidative damage of cellular macromolecules such as nuclear and mitochondrial DNA, membranes and proteins, thereby affecting major cell functions and cell survival (52–55). Moreover, there is an intimate link between adaptive changes in cell metabolism, generation of ROS, and the death threshold at the mitochondria by interconnected metabolic and redox sensitive pathways (56, 57). Therefore, the ability of the SLC25A1-inhibitor BTA to disrupt redox homeostasis makes it an attractive approach to enhance the cytotoxic effects of ROS-dependent treatments such as IR.

Finally, pharmacologic inhibition of SLC25A1 reduced the flexibility of oxic and anoxia-tolerant NCI-H460 cells to oxidize major metabolic fuels, which could in turn reduce their capability to meet altered nutrient availability after hypoxia-induced microenvironmental changes. Furthermore, BTA treatment decreased the capacity of NCI-H460 cells to oxidize FAs and also reduced FA uptake, particularly in anoxia-tolerant cells. Thereby our findings

corroborate data obtained by genetic knockout of SLC25A1 in NCI-H460 cells revealing increased glycolysis and the usage of glutamine to compensate for the loss of FA synthesis as a consequence of reduced citrate transport (44). The same group further highlighted the complex functional role of SLC25A1 in glycolysis, redox homeostasis, and lipogenesis by quantitative metabolic flux analysis (44). Of note, dependency on the uptake of FAs has been recognized as a specific metabolic vulnerability of Hx cancer cells (58, 59).

Our metabolic investigations demonstrate that in addition to ensure the export of citrate from the mitochondria to the cytosol, e.g., for FA synthesis, SLC25A1 seems to be crucial for mitochondrial homeostasis and increased metabolic flexibility of the anoxia-tolerant NCI-H460 cells. Thereby our data confirm the assumption that SLC25A1 might be crucial for metabolic plasticity of cancer cells enabling adaptation and survival under adverse conditions such as nutrient starvation (glucose) or oxidative stress suggested by others (39, 47). But our data extend the stress conditions to acute and chronic cycling severe hypoxia. It appears that inhibition of SLC25A1 leads to a massive disturbance of mitochondrial metabolism and this might severely affect the ability of cancer cells to cope with the toxic effects of IR leading to radiosensitization and enhanced clonogenic cell death.

However, radiosensitivity is largely determined by the ability of the cells to repair radiation-induced DNA DSB. Analyzing the time-dependent formation and resolution of γH2AX –positive DNA repair foci as a mean of DNA-DSB we found that BTA treatment did not alter the initial amount of radiation-induced γH2AX -foci at 0.5–1 h after irradiation. However, BTA-mediated SLC25A1 inhibition slowed the kinetics of γH2AX-foci resolution, and this resulted in significantly higher levels of residual DNA damage in oxic and anoxia-tolerant NCI-H460 cells exposed to combined treatment when compared to irradiation alone at 2, 4, 6, and 24 h after irradiation.

Unexpectedly, we found that despite the obvious differences in radiosensitivity between anoxia-tolerant and oxic control NCI-H460 cells the kinetics in formation and resolution of γH2AX-foci indicative were rather similar in both cell lines. Therefore, it was not surprising that both cell lines responded similarly to combined treatment with BTA and IR with respect to the kinetics of the induction and repair of DNA DSB. From these data we conclude that other parameters of the DNA damage response such as their ability to cope with radiation-induced ROS might dictate the adaptation-induced differences in radiosensitivity and that the resulting differences in the extent of radiosensitization between oxic control and anoxia-tolerant cells might be due to drug-induced interference with redox homeostasis and disturbance of mitochondrial metabolism to meet cellular energy demands during the radiation response.

Nevertheless, these observations revealed that inhibition of SLC25A1 impacts DNA repair. Analyzing the underlying mechanisms we found that BTA-mediated inhibition of DSB repair correlated with an increase in D2-hydroxyglutarate levels induced by BTA treatment. In this context, BTA treatment led to elevated levels of D-2-HG in both NCI-H460 oxic and anoxia-tolerant cancer cells. Thereby, pharmacologic inhibition of SLC25A1 with BTA reproduces another metabolic effect of genetic inhibition of SLC25A1 where somatic loss of SLC25A1 induced a broad dysregulation of mitochondrial metabolism with accumulation of L- and D-enantiomers of 2-HG in NCI-H460 cells (44). Moreover, deletion of the SLC25A1 gene in humans causes the neurometabolic disorder D- and L-2-hydroxyglutaric aciduria, which is also characterized by increased accumulation of 2-HG (60, 61). These observations make it highly likely that elevated D-2-HG levels observed in response to BTA treatment are a direct and acute consequence of SLC25A1 inhibition.

Both enantiomers of 2-HG have been shown to promote malignant progression by their inhibitory action on α-ketoglutarate (αKG)-dependent dioxygenases; they are either synthesized as so-called "oncometabolite" as a result of gain-of-function mutations in IDH1/IDH2 (62, 63) or as pathologic metabolites in Hx cancer cells (64, 65).

Of note, accumulation of 2-HG has recently been linked to inhibition of DNA repair by inducing a homologous recombination repair defect thereby sensitizing cancer cells to poly (ADP-ribose) polymerase (PARP) inhibitors (46, 66). Furthermore, 2-HG was also shown to inhibit ALKBH DNA repair enzymes, leading to enhanced sensitivity to alkylating agents (67). Our new findings strongly suggest that BTA-induced accumulation of 2-HG might contribute to BTA-mediated radiosensitization and this finding could be exploited therapeutically in combination treatments.

Taken together, we identified a role of SLC25A1-regulated redox homeostasis in the tolerance of cancer cells to acute and chronic cycling hypoxia and increased radioresistance caused by adaptation to these stress conditions. In addition, our results clearly demonstrate that inhibition of SLC25A1 by the small molecule inhibitors BTA and CNASB is suited to increase radiosensitivity of NCI-H460 cancer cells exposed to acute or chronic cycling hypoxia. Mechanistically, treatment with the SLC25A1-inhibitor BTA disturbed cellular redox homeostasis by decreasing NADPH/GSH levels but also affected mitochondrial metabolism and cellular energy provision thereby reducing cancer cell survival (summarized in **Figure 6F**). Importantly, we demonstrate that BTA treatment impaired the repair of IR-induced DNA DSB and this was associated with elevation of D-2-HG levels. Since the method allowed us only to detect D-2-HG we cannot exclude that BTA treatment might also increase L-2-HG levels.

The pronounced effects of the SLC25A1-inhibitor BTA on cellular antioxidant capacity, cell metabolism and DNA repair make SLC25A1 inhibitors such as BTA interesting leads for the development of metabolic inhibitors of radioresistance. However, the development of small molecule inhibitors specifically targeting SLC25A1 at nanomolar drug concentrations is required for clinical translation of this approach.

Importantly, high expression of SLC25A1 in lung cancer patients was associated with a poor outcome, particularly after successful surgery (R0-resection), pointing to a potential clinical relevance of SLC25A1 particularly in terms of tumor recurrence. Moreover, exposure to IR, acute hypoxia, and chronic cycling severe hypoxia increased SLC25A1 expression in our *in vitro* models of anoxia-tolerant cancer cells. These findings suggest that SLC25A1 could be relevant as a potential biomarker of increased antioxidant capacity and metabolic flexibility and thus of a high potential for metabolic escape from radio (chemo)therapy.

We conclude that targeting the increased metabolic flexibility of cancer cells with tolerance to environmental stress, or of metabolic escape mechanisms ensuring DNA repair and cell survival under therapy, e.g., by pharmacologic inhibition of SLC25A1, represent attractive strategies to enhance vulnerability to genotoxic treatments and overcome microenvironment-mediated resistance to radio(chemo)therapy in advanced cancers.

MATERIALS AND METHODS

Reagents and Cell Lines

If not stated otherwise, all chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). NCI-H460 lung adenocarcinoma cells, DU145 prostate cancer cells and T98G glioblastoma cells were obtained from ATCC (Bethesda, MD, USA) and were routinely tested for mycoplasma. NCI-H460, DU145 or T98G cells with tolerance to cycling severe hypoxia/reoxygenation stress were generated by exposure to 16 cycles (T98G) or 25 cycles (NCI-H460, DU145) of severe hypoxia (48 h, <0.1% O_2) and reoxygenation (120-h air plus 5% CO_2 referred as 20% O_2) as described earlier (24). These cells hypoxia/reoxygenation-tolerant cells will be termed "anoxia-tolerant cells" throughout the manuscript. Control cells were cultured in parallel under standard ambient O_2 conditions (20% O_2 plus 5% CO_2; the control cells will be termed "oxic cells" throughout the manuscript) (24, 59). Upon selection, cancer cells were routinely grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (Gibco/Life Technologies, Carlsbad, CA, USA) and maintained in a humidified incubator at 37°C and 5% CO_2 (referred to as "normoxia" or "Nx conditions,"). For severely Hx conditions cells were grown in a humidified hypoxia work station (*In vivo* 400, Ruskinn Technology Ltd., Bridgend, Great Britain) at 37°C, 0.2% O_2, and 5% CO_2 (referred to as "hypoxia" or "Hx conditions,").

Patient Survival Data

Patient array data were obtained from and analyzed by Kaplan–Meier plotter tool (kmplot.com) as described elsewhere (40, 41). The cohort was split by median of SLC25A1 expression ("High" and "Low," respectively). Analysis was performed in the cohort once without additional restrictions and once including only patients which had successful surgery (only surgical margins negative), as described in detail elsewhere (40). For further details of used settings please refer to Tables S1 and S2 in Supplementary Material.

qRT-PCR Analysis

cDNA was synthesized from 1 µg of total RNA using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Specific primers were synthesized based on available sequences for each listened gene. Primer sets for qRT-PCR were designed with the Blast web tool (U.S. National Center for Biotechnology Information, Bethesda, MD, USA). To exclude cross-reaction of primers with the genes the sequence of interest was compared as well with the Blast database. PCR products were 150–200 bp in size. We used published β2-microglobulin (B2M) primer sequences as housekeeping gene (68). qRT-PCR and cycling conditions were performed using specific oligonucleotide primers (*B2M* forward: TGCTGTCTCCATGTTTGATGTATCT; reverse: TCTCTGCTCCCCACCTCTAAGT; *SLC25A1* forward: CAACGGGGTGAGGGCAT; reverse: CTCGGTGGGGAAGGT GATG; *IDH1* forward: CTCTGTGGCCCAAGGGTATG; reverse: GGATTGGTGGACGTCTCCTG; IDH2 forward: CCTGCTCG TTCGCTCTCC; reverse: GCTTCGCCACCTTGATCCT) and using qPCR kit for SYBR® Green I, 6-Carboxyl-X-Rhodamine (ROX) (Eurogentec, Cologne, Germany) according to the manufacturers protocol. Reactions were carried out on an ABI Prism 7900HT using MicroAmp Optical 384 well Reaction plate (Applied Biosystems by Life Technologies, Bleijswijk, Netherlands) and BIO-RAD PCR Sealers Microseal "B" Film Adhesive seal (optically clear; BIORAD, Munich, Germany). Melting curves were obtained after each PCR run and showed single PCR products. cDNAs were run in triplicate, without reverse transcriptase and no-template controls were run in duplicates. Expression levels for the genes of interest and for the housekeeping gene *B2M* were measured in three independent PCR runs. Expression ratios were calculated using the geometric mean expression of the housekeeping gene *B2M* to normalize the expression data for the genes of interest according to the $2^{-\Delta\Delta Ct}$—method as described by others (69).

Western Blot Analysis

Anti-rabbit SLC25A1 Polyclonal antibody (Thermo Fisher, Rockford, IL, USA) and anti-mouse β-actin from Sigma Aldrich (St. Louis, MO, USA) were used for Western blot analysis. After harvesting, cells were lysed in 75 µL of RIPA buffer containing 0,5% Sodiumdesoxycholate, 1% NP-40 (Nonidet), 0,1% Sodiumdodecylsulfate (SDS), 50-mM Tris-HCl, 150-mM NaCl, 5-µg/mL aprotinin, 5-µg/mL leupeptin, and 3-µg/mL pepstatin. Protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred onto polyvinylidene fluoride (PVDF) membranes (Roth, Karlsruhe, Germany). Blots were blocked in RotiBlock (Roth, Karlsruhe, Germany) for 1 h at room temperature. The membranes were incubated overnight at 4°C with the respective primary antibodies. The secondary antibody was incubated for 1 h at room temperature. Detection of antibody binding was performed by enhanced chemiluminescence (ECL Western Blotting Analysis System; GE Healthcare/Amersham Biosciences, Freiburg, Germany). Equal loading was verified by antibodies against β-actin. Densitometry analysis was performed using ImageJ 2.00 (National Institutes of Health, Bethesda, MD, USA).

Determination of Redox Homeostasis

NADPH levels were measured using NADPH Glo Assay (Promega, Madison, WI, USA) according to manufacturer's protocol. Briefly, adherent cells in 96-well plates were lysed and heated under acidic and basic conditions to measure $NADP^+$ and NAPH individually using a luciferase-coupled enzymatic reaction. In parallel, technical replicates were fixed with 4% paraformaldehyde and stained with 10-µg/mL solution of fluorescent dye Hoechst 33342 (Thermo Scientific, Waltham, MA, USA) for normalization to DNA content. Luminescence and Flourescence were measured

in triplicates using a BioTek Synergy Microplate reader (BioTek, Winooski, NH, USA).

Levels of reduced Glutathione (GSH) were determined by using Monochlorobimane (MCB) which was described to be specific for GSH metabolized by Glutathione-S-Transferase, leading to a fluorescent adduct (70). The published protocol for measurement of GSH in a Microplate Reader (71) was adapted for using 10-µM MCB with 15 min of preincubation. To rule out alterations in the speed of dye metabolism instead of alterations in absolute cellular GSH levels, kinetic measurements of fluorescence were performed for 15 min at 37°C. Each assay contained cells treated with H_2O_2 to deplete GSH as a negative control.

To quantify mitochondrial ROS production, cells were stained for 30 min at 37°C with 5 µM of MitoSox (Molecular Probes/Invitrogen, Carlsbad, CA, USA). To quantify cellular ROS production, cells were stained for 30 min at 37°C with 5 µM of Dihydroethidium (DHE) (Molecular Probes/Invitrogen, Carlsbad, CA, USA). MitoSox- or DHE-positive cells were detected by flow cytometry (BD Accuri C6, Becton Dickinson, Heidelberg, Germany; FL-2). Fractions of gated positive cells (at least 10,000) with higher fluorescence were evaluated. Fold changes were quantified to the corresponding controls.

Extracellular Flux Analysis

NCI-H460 cells were plated at 7.500 cells/well in XF96 microplates (Seahorse Bioscience, Billerica, MA, USA) in RPMI Medium with 10% FCS according to manufacturer's recommendations 48 h prior to the assay. Treatment with 5 mM BTA was performed for 24 h. One hour prior to the assay, medium was exchanged to XF base medium (Seahorse Bioscience, Billerica, MA, USA) with 1-mM Pyruvate, 2-mM Glutamine and 10-mM Glucose and incubated at 37°C without CO_2. During assays, OCR was measured using a Seahorse XFe 96 analyzer. Mito Stress Test Kit containing 1-µM Oligomycin, 2-µM Carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (FCCP), 0.5-µM Rotenone and 0.5-µM Antimycin A was perfomed according to manufacturer's protocol. For individual normalization to DNA content, fluorescence was measured after cells were fixed with 4% PFA and stained with 10-µg/mL Hoechst 33342 solution after each assay. Fuel Flex Test Kit containing 3-µM BPTES, an inhibitor of glutaminase (GLS1), 4-µM etomoxir, an inhibitor of carnitine palmitoyl-transferase 1 A (CPT1A) and 2-µM UK5099, an inhibitor of the mitochondrial pyruvate carrier (MPC), was also performed according to manufacturer's protocol. Data were analyzed using Wave 2.4 software (Seahorse Bioscience, Billerica, MA, USA).

Determination of Fatty-Acid Uptake

The uptake of FA was quantified by using fluorescent FA analog C1-BODIPY® 500/510 C12. In brief, fluorescent FA (5 µM) were added 24 h after treatment with 5-mM BTA to serum-free media. We quenched the fluorescence of FA in media by adding trypan blue (0.33 mM) to the media. The uptake of fluorescent FA was measured after 1 h, at 37°C spectrophotometrically at 485/528 nm (59). For individual normalization to DNA content, Hoechst 33342 fluorescence was measured after the assay. Cells were fixed with 4% PFA and stained with 10-µg/mL Hoechst 33342 solution.

Quantification of D-2-Hydroxyglutarate

Levels of D-2-HG were measured using colorimetric D-2-Hydroxyglutarate Assay Kit (BioVision, Milpitas, CA, USA) according to manufacturer's protocol. At a glance, 10^7 cells were lysed, spun down and supernatant was transferred into a 96-well plate to quantify the enzymatic conversion of D-2-Hydroxyglutarate to α-Ketoglutarate leading to colored product which is detected photometrically at 450 nm using a BioTek Synergy Microplate reader (BioTek, Winooski, NH, USA).

Irradiation and Treatment

1,2,3-benzene-tricarboxylic acid and CNASB were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of 200-mM BTA were made with phosphate-buffered aqua bidest and 14-mM CNASB was solved in DMSO. Irradiation was performed at room temperature with an X-ray machine (Precision X-Ray Inc., North Branford, CT, USA) operated at 320 kV, 12.5 mA with a 1.65-mm Al filter, at a distance of 50 cm and a dose rate of 3.71 Gy/min. For an irradiation dose of 2 Gy, the cells were irradiated approximately 32 s, for 5 Gy approximately 81 s. Cells were returned to the incubator immediately after exposure to IR. For irradiation under Hx conditions, Hx cell dishes were kept in BD GasPak EZ Pouch System (Becton Dickinson, Heidelberg, Germany). In terms of combined treatments, inhibitors were added 2 h prior to irradiation.

Immunofluorescence: γH2AX Assays

Cells were seeded on glass coverslips placed in 12-well plates and irradiated 24 h later with 5 Gy. Next, cells were fixed and permeabilized in 3% PFA/0.2% Triton-X100 for 15 min and incubated in blocking solution including 2% goat serum at room temperature for 1 h. γH2AX foci were stained for 1 h at room temperature (RT) with Alexa Fluor® 647 mouse anti-H2A.X (pS139) (BD Biosciences, San Jose, CA, USA) diluted 1:50 in blocking solution. DNA was stained with Hoechst33342 (3 µM in PBS) for 30 min at RT. Coverslips were mounted onto glass slides with DAKO mounting medium (Dako NA Inc., Carpinteria, CA, USA). Slides were analyzed with a Zeiss Axiovert 200 fluorescence microscope with ApoTome and ZEN imaging software (Carl Zeiss, Goettingen, Germany). γH2AX foci in at least 50 cells per slide were counted with the Focinator software developed in our laboratory (72, 73).

Colony Formation Assays

Clonogenic cell survival in response to the respective treatments was determined comparing the clonogenic survival of cells cultured under Nx and severely Hx conditions. For treatment in normoxia, exponentially growing cells were seeded in tissue culture flasks, incubated under standard culturing conditions (20% O_2, 5% CO_2, 37°C) and irradiated 24 h later (0 to 5 Gy) without or with prior BTA treatment (5 mM). BTA treatment was performed 2 h prior to irradiation. For treatment in hypoxia, tissue culture flasks of exponentially growing cells were exposed to severe hypoxia (0.2% O_2) 2 h prior to BTA treatment and 4 h prior to irradiation, respectively. After completion of the treatments, cells were incubated for 24 h under Nx or Hx conditions, respectively,

then washed, collected (0.05% Trypsin, 0.01% EDTA), and plated to 6-well plates at densities of 200–3,200 cells per well (delayed plating). The cell viability was checked before plating the cells by using CASY COUNT (Omni Life Science, Bremen, Germany) and only the number of viable cells was plated. Plates were subsequently incubated for 9 days under standard Nx conditions before quantification of colony formation. For this, cells were fixed in 3.7% formaldehyde and 70% ethanol, stained with 0.05% Coomassie blue, and colonies of at least 50 cells were counted by GelCount (Oxford Optronix, Oxfordshire, Great Britain). The plating efficiency and surviving fraction (SF) to corresponding Nx and Hx controls were calculated as described elsewhere (74).

Toxicity Testing

For quantification of apoptotic DNA-fragmentation (sub-G1 population), cells were incubated for 30 min at room temperature with a staining solution containing 50-µg/mL PI in a hypotonic citrate buffer 0.1% (w/v) sodium citrate and 0.05% (v/v) Triton X-100 and subsequently analyzed by flow cytometry (BD Accuri C6, Becton Dickinson, Heidelberg, Germany; FL-2) (75).

For determination of cell proliferation and viability, cells were washed with PBS (1x), fixed with Glutaraldehyde (0.1% in PBS), and stained with crystal violet (0.1% in PBS). The dye was released by TritonX-100 (0.2% in PBS) and measured spectrophotometrically at 540 nm as described elsewhere (76).

Statistics

Data represent mean values of at least three independent experiments ± SEM except for **Figures 5A,B** which show data from $n = 12–18$ wells from two independent experiments. Data analysis was performed either by two-way ANOVA test using parametric methods and employing Tukey multiple comparison post-test where appropriate or by unpaired Student's *t*-test using Prism6 software (Graph Pad Inc., La Jolla, CA, USA). The values of $P \leq 0.05$ were considered significant.

AUTHOR CONTRIBUTIONS

JM, JH, and VJ designed and conceptualized the research. JH, CH, and JM performed experiments, analyzed, validated, and visualized the results. JM, JH, and VJ wrote the original manuscript draft. JM and VJ supervised the work. VJ acquired the funding. All authors critically revised, edited, and approved the final version of the manuscript.

ACKNOWLEDGMENTS

We thank A. Warda for excellent technical support, K. Al-Refae and A. Krysztofiak for support with DNA DSB foci analysis, and the Focinator-Software and the Brigitte und Dr. Konstanze Wegener-Stiftung for funding of the Seahorse XFe 96 analyzer to VJ.

FUNDING

The work was supported by grants of the German Research Association (grant number DFG GRK1739/2) and the Deutsche Krebshilfe/Mildred-Scheel-Stiftung (grant numbers 110355 and 70112711) to VJ.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fonc.2018.00170/full#supplementary-material.

REFERENCES

1. Salem A, Asselin MC, Reymen B, Jackson A, Lambin P, West CML, et al. Targeting hypoxia to improve non-small cell lung cancer outcome. *J Natl Cancer Inst* (2018) 110(1):14–30. doi:10.1093/jnci/djx160
2. Barcellos-Hoff MH, Lyden D, Wang TC. The evolution of the cancer niche during multistage carcinogenesis. *Nat Rev Cancer* (2013) 13(7):511–8. doi:10.1038/nrc3536
3. Chiche J, Brahimi-Horn MC, Pouyssegur J. Tumour hypoxia induces a metabolic shift causing acidosis: a common feature in cancer. *J Cell Mol Med* (2010) 14(4):771–94. doi:10.1111/j.1582-4934.2009.00994.x
4. Chen JL, Merl D, Peterson CW, Wu J, Liu PY, Yin H, et al. Lactic acidosis triggers starvation response with paradoxical induction of TXNIP through MondoA. *PLoS Genet* (2010) 6(9):e1001093. doi:10.1371/journal.pgen.1001093
5. Gatenby RA, Gillies RJ. A microenvironmental model of carcinogenesis. *Nat Rev Cancer* (2008) 8(1):56–61. doi:10.1038/nrc2255
6. Flavahan WA, Gaskell E, Bernstein BE. Epigenetic plasticity and the hallmarks of cancer. *Science* (2017) 357(6348):eaal2380. doi:10.1126/science.aal2380
7. Macaluso M, Paggi MG, Giordano A. Genetic and epigenetic alterations as hallmarks of the intricate road to cancer. *Oncogene* (2003) 22(42):6472–8. doi:10.1038/sj.onc.1206955
8. Chan DA, Giaccia AJ. Hypoxia, gene expression, and metastasis. *Cancer Metastasis Rev* (2007) 26(2):333–9. doi:10.1007/s10555-007-9063-1
9. Harris AL. Hypoxia—a key regulatory factor in tumour growth. *Nat Rev Cancer* (2002) 2(1):38–47. doi:10.1038/nrc704
10. Vaupel P. Tumor microenvironmental physiology and its implications for radiation oncology. *Semin Radiat Oncol* (2004) 14(3):198–206. doi:10.1016/j.semradonc.2004.04.008
11. Rankin EB, Giaccia AJ. Hypoxic control of metastasis. *Science* (2016) 352(6282):175–80. doi:10.1126/science.aaf4405
12. Bristow RG, Hill RP. Hypoxia and metabolism. Hypoxia, DNA repair and genetic instability. *Nat Rev Cancer* (2008) 8(3):180–92. doi:10.1038/nrc2344
13. Liauw SL, Connell PP, Weichselbaum RR. New paradigms and future challenges in radiation oncology: an update of biological targets and technology. *Sci Transl Med* (2013) 5(173):173sr2. doi:10.1126/scitranslmed.3005148
14. Wouters BG, Koritzinsky M. Hypoxia signalling through mTOR and the unfolded protein response in cancer. *Nat Rev Cancer* (2008) 8(11):851–64. doi:10.1038/nrc2501
15. Lee CT, Boss MK, Dewhirst MW. Imaging tumor hypoxia to advance radiation oncology. *Antioxid Redox Signal* (2014) 21(2):313–37. doi:10.1089/ars.2013.5759
16. Ljungkvist ASE, Bussink J, Kaanders JH, Wiedenmann NE, Vlasman R, van der Kogel AJ. Dynamics of hypoxia, proliferation and apoptosis after irradiation in a murine tumor model. *Radiat Res* (2006) 165(3):326–36. doi:10.1667/RR3515.1
17. Matsumoto S, Yasui H, Mitchell JB, Krishna MC. Imaging cycling tumor hypoxia. *Cancer Res* (2010) 70(24):10019–23. doi:10.1158/0008-5472.CAN-10-2821
18. Vaupel P, Harrison L. Tumor hypoxia: causative factors, compensatory mechanisms, and cellular response. *Oncologist* (2004) 9(Suppl 5):4–9. doi:10.1634/theoncologist.9-90005-4
19. Chou CW, Wang CC, Wu CP, Lin YJ, Lee YC, Cheng YW, et al. Tumor cycling hypoxia induces chemoresistance in glioblastoma multiforme by upregulating the expression and function of ABCB1. *Neuro Oncol* (2012) 14(10):1227–38. doi:10.1093/neuonc/nos195
20. Span PN, Bussink J. Biology of hypoxia. *Semin Nucl Med* (2015) 45(2):101–9. doi:10.1053/j.semnuclmed.2014.10.002

21. Tellier C, Desmet D, Petit L, Finet L, Graux C, Raes M, et al. Cycling hypoxia induces a specific amplified inflammatory phenotype in endothelial cells and enhances tumor-promoting inflammation in vivo. *Neoplasia* (2015) 17(1): 66–78. doi:10.1016/j.neo.2014.11.003
22. Weinmann M, Belka C, Guner D, Goecke B, Muller I, Bamberg M, et al. Array-based comparative gene expression analysis of tumor cells with increased apoptosis resistance after hypoxic selection. *Oncogene* (2005) 24(38):5914–22. doi:10.1038/sj.onc.1208748
23. Weinmann M, Jendrossek V, Guner D, Goecke B, Belka C. Cyclic exposure to hypoxia and reoxygenation selects for tumor cells with defects in mitochondrial apoptotic pathways. *FASEB J* (2004) 18(15):1906–8. doi:10.1096/fj.04-1918fje
24. Matschke J, Riffkin H, Klein D, Handrick R, Ludemann L, Metzen E, et al. Targeted inhibition of glutamine-dependent glutathione metabolism overcomes death resistance induced by chronic cycling hypoxia. *Antioxid Redox Signal* (2016) 25(2):89–107. doi:10.1089/ars.2015.6589
25. Dewhirst MW. Intermittent hypoxia furthers the rationale for hypoxia-inducible factor-1 targeting. *Cancer Res* (2007) 67(3):854–5. doi:10.1158/0008-5472.CAN-06-4744
26. Song C, Hong BJ, Bok S, Lee CJ, Kim YE, Jeon SR, et al. Real-time tumor oxygenation changes after single high-dose radiation therapy in orthotopic and subcutaneous lung cancer in mice: clinical implication for stereotactic ablative radiation therapy schedule optimization. *Int J Radiat Oncol Biol Phys* (2016) 95(3):1022–31. doi:10.1016/j.ijrobp.2016.01.064
27. Levine AJ, Puzio-Kuter AM. The control of the metabolic switch in cancers by oncogenes and tumor suppressor genes. *Science* (2010) 330(6009):1340–4. doi:10.1126/science.1193494
28. Rouschop KM, Dubois LJ, Keulers TG, van den Beucken T, Lambin P, Bussink J, et al. PERK/eIF2alpha signaling protects therapy resistant hypoxic cells through induction of glutathione synthesis and protection against ROS. *Proc Natl Acad Sci U S A* (2013) 110(12):4622–7. doi:10.1073/pnas.1210633110
29. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) 144(5):646–74. doi:10.1016/j.cell.2011.02.013
30. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. *Nat Rev Cancer* (2011) 11(2):85–95. doi:10.1038/nrc2981
31. DeBerardinis RJ, Chandel NS. Fundamentals of cancer metabolism. *Sci Adv* (2016) 2(5):e1600200. doi:10.1126/sciadv.1600200
32. Nakashima R, Goto Y, Koyasu S, Kobayashi M, Morinibu A, Yoshimura M, et al. UCHL1-HIF-1 axis-mediated antioxidant property of cancer cells as a therapeutic target for radiosensitization. *Sci Rep* (2017) 7(1):6879. doi:10.1038/s41598-017-06605-1
33. Kuo ML, Sy AJ, Xue L, Chi M, Lee MT, Yen T, et al. RRM2B suppresses activation of the oxidative stress pathway and is up-regulated by p53 during senescence. *Sci Rep* (2012) 2:822. doi:10.1038/srep00822
34. Deponte M. The incomplete glutathione puzzle: just guessing at numbers and figures? *Antioxid Redox Signal* (2017) 27(15):1130–61. doi:10.1089/ars.2017.7123
35. Toledano MB, Huang ME. The unfinished puzzle of glutathione physiological functions, an old molecule that still retains many enigmas. *Antioxid Redox Signal* (2017) 27(15):1127–9. doi:10.1089/ars.2017.7230
36. Jiang L, Shestov AA, Swain P, Yang C, Parker SJ, Wang QA, et al. Reductive carboxylation supports redox homeostasis during anchorage-independent growth. *Nature* (2016) 532(7598):255–8. doi:10.1038/nature17393
37. Palmieri EM, Spera I, Menga A, Infantino V, Porcelli V, Iacobazzi V, et al. Acetylation of human mitochondrial citrate carrier modulates mitochondrial citrate/malate exchange activity to sustain NADPH production during macrophage activation. *Biochim Biophys Acta* (2015) 1847(8):729–38. doi:10.1016/j.bbabio.2015.04.009
38. Palmieri F. The mitochondrial transporter family (SLC25): physiological and pathological implications. *Pflugers Arch* (2004) 447(5):689–709. doi:10.1007/s00424-003-1099-7
39. Catalina-Rodriguez O, Kolukula VK, Tomita Y, Preet A, Palmieri F, Wellstein A, et al. The mitochondrial citrate transporter, CIC, is essential for mitochondrial homeostasis. *Oncotarget* (2012) 3(10):1220–35. doi:10.18632/oncotarget.714
40. Gyorffy B, Surowiak P, Budczies J, Lanczky A. Online survival analysis software to assess the prognostic value of biomarkers using transcriptomic data in non-small-cell lung cancer. *PLoS One* (2013) 8(12):e82241. doi:10.1371/journal.pone.0082241
41. Gyorffy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q, et al. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. *Breast Cancer Res Treat* (2010) 123(3):725–31. doi:10.1007/s10549-009-0674-9
42. Kolukula VK, Sahu G, Wellstein A, Rodriguez OC, Preet A, Iacobazzi V, et al. SLC25A1, or CIC, is a novel transcriptional target of mutant p53 and a negative tumor prognostic marker. *Oncotarget* (2014) 5(5):1212–25. doi:10.18632/oncotarget.1831
43. Aluvila S, Sun J, Harrison DH, Walters DE, Kaplan RS. Inhibitors of the mitochondrial citrate transport protein: validation of the role of substrate binding residues and discovery of the first purely competitive inhibitor. *Mol Pharmacol* (2010) 77(1):26–34. doi:10.1124/mol.109.058750
44. Jiang L, Boufersaoui A, Yang C, Ko B, Rakheja D, Guevara G, et al. Quantitative metabolic flux analysis reveals an unconventional pathway of fatty acid synthesis in cancer cells deficient for the mitochondrial citrate transport protein. *Metab Eng* (2017) 43(Pt B):198–207. doi:10.1016/j.ymben.2016.11.004
45. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem* (1998) 273(10):5858–68. doi:10.1074/jbc.273.10.5858
46. Sulkowski PL, Corso CD, Robinson ND, Scanlon SE, Purshouse KR, Bai H, et al. 2-Hydroxyglutarate produced by neomorphic IDH mutations suppresses homologous recombination and induces PARP inhibitor sensitivity. *Sci Transl Med* (2017) 9(375):eaal2463. doi:10.1126/scitranslmed.aal2463
47. Albanese C, Avantaggiati ML. The SLC25A1-p53 mutant crosstalk. *Aging (Albany NY)* (2015) 7(8):519–20. doi:10.18632/aging.100785
48. Wise DR, Ward PS, Shay JE, Cross JR, Gruber JJ, Sachdeva UM, et al. Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of alpha-ketoglutarate to citrate to support cell growth and viability. *Proc Natl Acad Sci U S A* (2011) 108(49):19611–6. doi:10.1073/pnas.1117773108
49. Infantino V, Iacobazzi V, Menga A, Avantaggiati ML, Palmieri F. A key role of the mitochondrial citrate carrier (SLC25A1) in TNFalpha- and IFNgamma-triggered inflammation. *Biochim Biophys Acta* (2014) 1839(11):1217–25. doi:10.1016/j.bbagrm.2014.07.013
50. Lieburg-Apers DC, Willems PH, Koopman WJ, Grefte S. Interactions between mitochondrial reactive oxygen species and cellular glucose metabolism. *Arch Toxicol* (2015) 89(8):1209–26. doi:10.1007/s00204-015-1520-y
51. Ye J, Fan J, Venneti S, Wan YW, Pawel BR, Zhang J, et al. Serine catabolism regulates mitochondrial redox control during hypoxia. *Cancer Discov* (2014) 4(12):1406–17. doi:10.1158/2159-8290.CD-14-0250
52. Latorre-Pellicer A, Moreno-Loshuertos R, Lechuga-Vieco AV, Sanchez-Cabo F, Torroja C, Acin-Perez R, et al. Mitochondrial and nuclear DNA matching shapes metabolism and healthy ageing. *Nature* (2016) 535(7613):561–5. doi:10.1038/nature18618
53. Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. *Curr Biol* (2014) 24(10):R453–62. doi:10.1016/j.cub.2014.03.034
54. Sullivan LB, Chandel NS. Mitochondrial reactive oxygen species and cancer. *Cancer Metab* (2014) 2:17. doi:10.1186/2049-3002-2-17
55. Sato A, Nakada K, Hayashi J. Mitochondrial dynamics and aging: mitochondrial interaction preventing individuals from expression of respiratory deficiency caused by mutant mtDNA. *Biochim Biophys Acta* (2006) 1763 (5–6):473–81. doi:10.1016/j.bbamcr.2006.03.001
56. Fulda S, Galluzzi L, Kroemer G. Targeting mitochondria for cancer therapy. *Nat Rev Drug Discov* (2010) 9(6):447–64. doi:10.1038/nrd3137
57. Wu CC, Bratton SB. Regulation of the intrinsic apoptosis pathway by reactive oxygen species. *Antioxid Redox Signal* (2013) 19(6):546–58. doi:10.1089/ars.2012.4905
58. Kamphorst JJ, Cross JR, Fan J, de Stanchina E, Mathew R, White EP, et al. Hypoxic and Ras-transformed cells support growth by scavenging unsaturated fatty acids from lysophospholipids. *Proc Natl Acad Sci U S A* (2013) 110(22):8882–7. doi:10.1073/pnas.1307237110
59. Matschke J, Wiebeck E, Hurst S, Rudner J, Jendrossek V. Role of SGK1 for fatty acid uptake, cell survival and radioresistance of NCI-H460 lung cancer cells exposed to acute or chronic cycling severe hypoxia. *Radiat Oncol* (2016) 11:75. doi:10.1186/s13014-016-0647-1
60. Eguchi M, Ozaki E, Yamauchi T, Ohta M, Higaki T, Masuda K, et al. Manifestation of recessive combined D-2-, L-2-hydroxyglutaric aciduria in combination with 22q11.2 deletion syndrome. *Am J Med Genet A* (2018) 176(2):351–8. doi:10.1002/ajmg.a.38578

61. Pop A, Williams M, Struys EA, Monne M, Jansen EEW, De Grassi A, et al. An overview of combined D-2- and L-2-hydroxyglutaric aciduria: functional analysis of CIC variants. *J Inherit Metab Dis* (2018) 41(2):169–80. doi:10.1007/s10545-017-0106-7
62. Dang L, White DW, Gross S, Bennett BD, Bittinger MA, Driggers EM, et al. Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature* (2009) 462(7274):739–44. doi:10.1038/nature08617
63. Losman JA, Kaelin WG Jr. What a difference a hydroxyl makes: mutant IDH, (R)-2-hydroxyglutarate, and cancer. *Genes Dev* (2013) 27(8):836–52. doi:10.1101/gad.217406.113
64. Intlekofer AM, Dematteo RG, Venneti S, Finley LW, Lu C, Judkins AR, et al. Hypoxia induces production of L-2-hydroxyglutarate. *Cell Metab* (2015) 22(2):304–11. doi:10.1016/j.cmet.2015.06.023
65. Oldham WM, Clish CB, Yang Y, Loscalzo J. Hypoxia-mediated increases in L-2-hydroxyglutarate coordinate the metabolic response to reductive stress. *Cell Metab* (2015) 22(2):291–303. doi:10.1016/j.cmet.2015.06.021
66. Gagne M, Boulay K, Topisirovic I, Huot ME, Mallette FA. Oncogenic activities of IDH1/2 mutations: from epigenetics to cellular signaling. *Trends Cell Biol* (2017) 27(10):738–52. doi:10.1016/j.tcb.2017.06.002
67. Wang P, Wu J, Ma S, Zhang L, Yao J, Hoadley KA, et al. Oncometabolite D-2-hydroxyglutarate inhibits ALKBH DNA repair enzymes and sensitizes IDH mutant cells to alkylating agents. *Cell Rep* (2015) 13(11):2353–61. doi:10.1016/j.celrep.2015.11.029
68. Caradec J, Sirab N, Keumeugni C, Moutereau S, Chimingqi M, Matar C, et al. 'Desperate house genes': the dramatic example of hypoxia. *Br J Cancer* (2010) 102(6):1037–43. doi:10.1038/sj.bjc.6605573
69. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* (2001) 29(9):e45. doi:10.1093/nar/29.9.e45
70. Rice GC, Bump EA, Shrieve DC, Lee W, Kovacs M. Quantitative analysis of cellular glutathione by flow cytometry utilizing monochlorobimane: some applications to radiation and drug resistance in vitro and in vivo. *Cancer Res* (1986) 46(12 Pt 1):6105–10.
71. Cox DP, Cardozo-Pelaez F. High throughput method for assessment of cellular reduced glutathione in mammalian cells. *J Environ Prot Sci* (2007) 1:23–8.
72. Oeck S, Malewicz NM, Hurst S, Al-Refae K, Krysztofiak A, Jendrossek V. The Focinator v2-0—graphical interface, four channels, colocalization analysis and cell phase identification. *Radiat Res* (2017) 188(1):114–20. doi:10.1667/RR14746.1
73. Oeck S, Malewicz NM, Hurst S, Rudner J, Jendrossek V. The Focinator—a new open-source tool for high-throughput foci evaluation of DNA damage. *Radiat Oncol* (2015) 10:163. doi:10.1186/s13014-015-0453-1
74. Franken NA, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells in vitro. *Nat Protoc* (2006) 1(5):2315–9. doi:10.1038/nprot.2006.339
75. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* (1991) 139(2):271–9. doi:10.1016/0022-1759(91)90198-O
76. Feoktistova M, Geserick P, Leverkus M. Crystal violet assay for determining viability of cultured cells. *Cold Spring Harb Protoc* (2016) 2016(4):343–6. doi:10.1101/pdb.prot087379

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 29 May 2018
doi: 10.3389/fonc.2018.00189

Hypoxic Signalling in Tumour Stroma

*Anu Laitala and Janine T. Erler**

Biotech Research and Innovation Centre (BRIC), University of Copenhagen (UCPH), Copenhagen, Denmark

Hypoxia is a common feature in solid tumors and is associated with cancer progression. The main regulators of the hypoxic response are hypoxia-inducible transcription factors (HIFs) that guide the cellular adaptation to hypoxia by gene activation. The actual oxygen sensing is performed by HIF prolyl hydroxylases (PHDs) that under normoxic conditions mark the HIF-α subunit for degradation. Cancer progression is not regulated only by the cancer cells themselves but also by the whole tumor microenvironment, which consists of cellular and extracellular components. Hypoxic conditions also affect the stromal compartment, where stromal cells are in close contact with the cancer cells. The important function of HIF in cancer cells has been shown by many animal models and described in hundreds of reviews, but less in known about PHDs and even less PHDs in stromal cells. Here, we review hypoxic signaling in tumors, mainly in the tumor stroma, with a focus on HIFs and PHDs.

Keywords: hypoxia, HIF, PHDs, tumour stroma, metastasis

OPEN ACCESS

Edited by:
Giuseppe Filomeni, Danish Cancer Society, Denmark

Reviewed by:
Kasper Rouschop, Maastricht University, Netherlands
Simone Cardaci, University of Glasgow, United Kingdom

***Correspondence:**
Janine T. Erler
janine.erler@bric.ku.dk

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *14 February 2018*
Accepted: *14 May 2018*
Published: *29 May 2018*

Citation:
Laitala A and Erler JT (2018) Hypoxic Signalling in Tumour Stroma. Front. Oncol. 8:189. doi: 10.3389/fonc.2018.00189

INTRODUCTION

Solid tumors are most often partly hypoxic. During the rapid growth of cancer cells, the surrounding vasculature becomes inadequate and is unable to meet the high demand of oxygen creating heterogeneously distributed hypoxic areas within the tumor. Hypoxia in tumors promotes abnormal angiogenesis, desmoplasia, and inflammation. It also boosts the selection of cancer cells that have a more malignant phenotype promoting tumor progression and metastasis, and thereby serving as an indicator for disease outcome. Hypoxic tumor cells are also resistant to radiotherapy and most chemotherapies. Tumors include stromal cells and extracellular matrix (ECM) in addition to cancer cells, which are also affected by the hypoxic environment (1, 2). An ever-increasing number of studies have highlighted the importance of the tumor microenvironment (TME) in regulating tumor progression and dissemination (3).

The cellular response to the drop in oxygen concentration leads to stabilization of hypoxia-inducible transcription factor (HIF) in all cell types, which is one of the key regulators of hypoxia response. The HIF prolyl hydroxylases (PHDs) are oxygen-dependent enzymes that target HIF for degradation in air. Thus, in hypoxia, PHDs are inactive, and HIF is stabilized. HIF regulates the expression of many genes that help cells to adapt to hypoxic conditions by decreasing oxygen consumption and increasing its supply. Generally, this includes shifting the energy metabolism to the less oxygen requiring glycolytic pathway and stimulation of the angiogenic genes to increase vascular flow to the hypoxic regions (4).

In addition to HIF activation, responses to hypoxia are also mediated through HIF-independent pathways. These signaling mechanisms include the unfolded protein response (UPR) and mammalian target of rapamycin (mTOR) signaling (5). They work as independent pathways, but in many cases, their signaling is integrated with HIF activation as well as with each other (6).

Unfolded protein response is activated during endoplasmic reticulum (ER) stress. ER stress arises from accumulation of unfolded proteins, which can be contributed by several factors such

as nutrient and calcium depletion and hypoxia. It consists of different and complex signal-transduction cascades, where pancreatic ER kinase (PERK), inositol-requiring enzyme 1, and activating transcription factor 6 act as sensor proteins, situated in the ER membrane. These mediate pro-survival signaling aiming to counteract ER stress by limiting the production of unfolded proteins and degrading the misfolded proteins preventing cellular damage (5, 7). However, if UPR is not able to re-establish ER homeostasis, it can also lead to pro-death signaling leading to apoptosis. Many publications have shown UPR to be activated in numerous tumor types where hypoxia is one of the promoting factors. Cancer cells have, however, found ways to avoid the ER stress-induced apoptosis (7). UPR signaling has been shown to target vascular endothelial growth factor (VEGF) in cancer cells. Activation of transcription factor 4, which is downstream of the PERK, was shown to induce VEGF mRNA levels, but to lesser extent than HIF signaling. UPR was, nevertheless, also shown to phosphorylate HIF-1α enhancing its activity, which resulted in increased VEGF expression (8). URP signaling has also been shown to have a role in tumor infiltrating immune cells, which can promote both immunosurveillance and immune escape mechanisms (9).

The mTOR kinase is a part of the mTOR complexes 1 and 2 (mTORC1 and 2), and it regulates cell survival, growth, and metabolism. mTOR mediates signals from different intracellular and extracellular stimuli such as growth factors, nutrients, stress, and oxygen levels (10). As a result, mTOR is phosphorylated at multiple sites, which in turn leads to direct phosphorylation of downstream targets such as eukaryotic translation initiation factor 4E binding protein 1 and ribosomal protein S6 kinase. Many of mTOR upstream signaling pathways go through the tumor suppressor tuberous sclerosis 1 and 2 (TSC1/2) complex, and it is also subject to hypoxic regulation. TSC1/2 complex negatively regulates the small GTPase ras-homolog-enriched-in-brain (Rheb). During mTOR activating signaling, TSC1/2 complex is inhibited by phosphorylation, leading to mTOR activation through active Rheb (6, 11). Hypoxia is known to increase the ratio of AMP/ATP, which leads to activation of AMP-activated protein kinase (AMPK). AMPK is capable of phosphorylating TSC2, but this phosphorylation on the contrary activates the TSC1/2 complex, which inhibits Rheb. Inactive Rheb in turn cannot phosphorylate mTOR, which attenuates the downstream signaling (12). mTOR signaling also intercepts with HIF-dependent hypoxic signaling. HIF upregulates the expression of REDD1 (regulated in development and DNA damage responses), which also activates TSC1/2 complex decreasing mTOR signaling (13). However, this inhibitory mechanism seems to have cell type-dependent differences (14). Oncogenic mTOR has also been reported to promote HIF signaling as well as HIF target gene expression such as lysyl oxidase (LOX) (15) in an HIF-dependent way. VEGF, however, has been shown to be regulated by both HIF-dependent and HIF-independent mechanisms (16). This was also seen in cancer-associated fibroblasts (CAFs) isolated from human breast cancers where tumor suppressor $p16^{INK4A}$ downregulation led to increased Akt/mTOR signaling also affecting HIF-α positively and further increasing the VEGF-A secretion. Enhanced Akt/mTOR signaling in CAFs was shown also to increase their invasion and migration capabilities. However, the role of HIF was not addressed (17).

Of these three different hypoxia response pathways mentioned, we will focus on the HIF signaling pathway that influences both cancer and stromal cells. Little is known about how HIF/PHDs activities regulate stromal cell function in the TME. Here, we review how HIF/PHD pathways impact on cancer progression in the different cellular and non-cellular compartments of TME.

HIFs AND PHDs: KEY PLAYERS IN OXYGEN SENSING AND HYPOXIA SIGNALING

Hypoxic signaling is mediated *via* the hypoxia-inducible transcription factor (HIF), which is a dimer consisting of two subunits, HIF-α and HIF-β. HIF-β (also known as ARNT) is constitutively expressed and stabile, but HIF-α is targeted to proteasomal degradation under normoxic conditions. Both subunits contain basic helix-loop-helix and PER-ARNT-SIM domains that are important for DNA binding and dimerization, but only HIF-α contains an oxygen-dependent degradation (ODD) domain that is important for the oxygen-dependent regulation. Under hypoxic conditions, HIF-α is able to bind with HIF-β forming a dimer that specifically binds to hypoxia response elements and so activates the transcription of over hundreds of genes that help the cells to adapt to low oxygen levels (18–21).

HIF-α has three different isoforms, HIF-1α, HIF-2α, and HIF-3α, of which the two first are more widely studied. HIF-1α and HIF-2α resemble each other, but the main differences are in their N-terminal transactivation domain (N-TAD). They both have a similar C-terminal transactivation domain (C-TAD), which contributes to the transcription of their shared targets, whereas the N-TAD differences confer the different binding capabilities and specificity among their targets (22). HIF-3α is the most different from the three and is subject to extensive alternative splicing resulting in many splicing variants. It has only one specific proline residue in the ODD domain and is lacking the C-TAD. Its function only as a transcription factor has been doubted and in fact different HIF-3α variants have been shown to have inductive and suppressive effects on HIF targets (23, 24).

The ODD domain contains two specific proline residues (Pro402 and 564 in human HIF-1α) that are hydroxylated under normoxic conditions by the HIF prolyl hydroxylase 1–3 (also known as EGLN2, 1, and 3 or HIF-P4H1-3) (**Figure 1**). These enzymes belong to the 2-oxoglutarate (2-OG)-dependent dioxygenases and require oxygen, iron, ascorbate, and 2-OG for the hydroxylation reaction. The hydroxylated prolines act as recognition sites for the von Hippel-Lindau (pVHL)–E3 ubiquitin ligase complex, which tags HIF-α for proteasomal degradation (18, 25, 26). HIF is also regulated by factor-inhibiting HIF (FIH) in normoxia. FIH belongs to the same 2-OG family as PHDs. It hydroxylates the asparagine residue within the C-TAD blocking HIF from binding to the p300-CBP coactivators and inhibits the transcriptional activation of HIF (27).

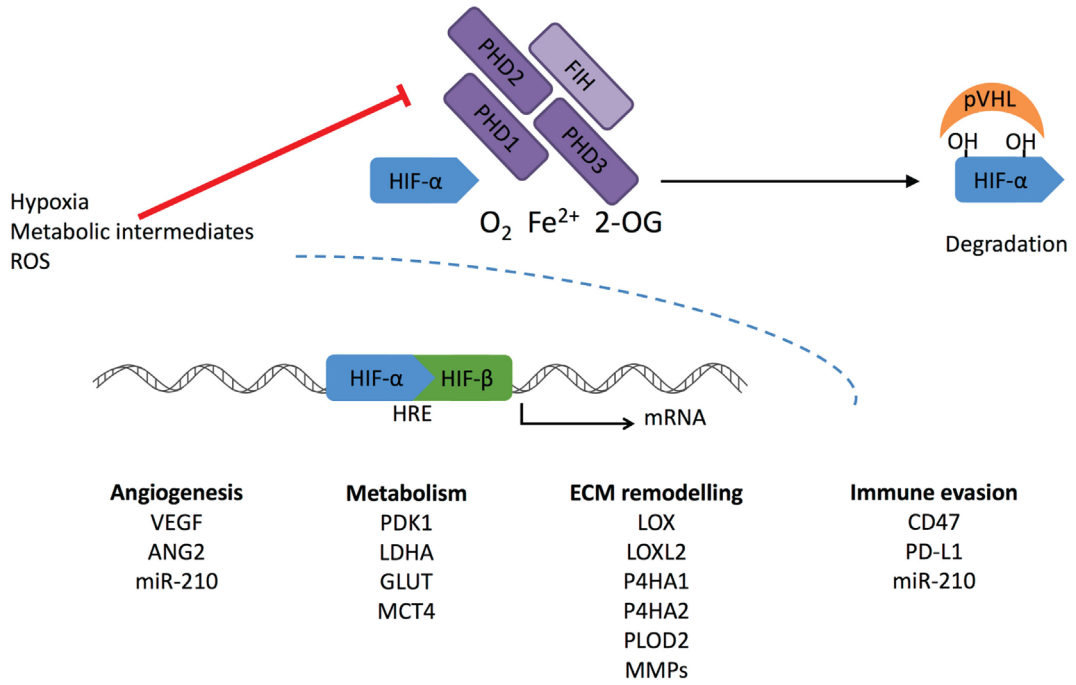


FIGURE 1 | Regulation of HIF-α stabilization. Cellular oxygen levels are sensed by HIF prolyl hydroxylase (PHD) 1–3. They hydroxylate two specific proline residues in the HIF-α subunit, which require O_2, Fe^{2+}, and 2-oxoglutarate (2-OG). 4-Hydroxyprolines are recognized by von Hippel-Lindau (pVHL) complex that ubiquitylates HIF-α causing its proteasomal degradation. Another member of the dioxygenase superfamily, factor-inhibiting HIF (FIH), hydroxylates one asparaginyl residue at the transcriptional domain of HIF causing suppression of the transcriptional activity. Under hypoxic conditions, PHD and FIH activity is inhibited, and unhydroxylated HIF-α forms a complex with HIF-β and activates transcription of HIF target genes. Reactive oxygen species (ROS) and intracellular metabolites can inhibit PHDs and FIH-stimulating transcriptional activation of HIF-1α, leading to increased expression under normoxic conditions.

HIF prolyl hydroxylases are fast in responding are fast in responding to changes in O_2 concentration due to their much higher K_m values than the physiological O_2 concentrations (28). They are widely expressed in different tissues, but at varying levels. PHD2 is the main enzyme to regulate HIF. Inactivation of PHD2 alone leads to HIF stabilization in normoxia. Both PHD1 and PHD3 are generally regarded to have a complementary role as inactivation of these alone does not lead to HIF stabilization (29). Global inactivation of PHDs in mice supports the finding that PHD2 is a key modulator in HIF regulation. Inactivation of PHD2 led to embryonic lethality, whereas phenotypes resulting from PHD1 or PHD3 inactivation were viable and grossly normal (30). PHDs have specificity toward different HIF-α hydroxylations. PHD2 preferentially hydroxylates HIF-1α, whereas PHD3 has a preference toward HIF-2α (31). Both PHD2 and PHD3 are hypoxia inducible, and it seems PHD2 is upregulated more by HIF-1 and PHD3 by HIF-2 (32). PHD2 has been reported to bind HIF-1α also in hypoxia inhibiting the HIF-1α N-terminal transcriptional activity without affecting its proteolysis (33).

HIF prolyl hydroxylases have been reported to have additional targets and to participate in HIF-independent signaling. PHD2 has been shown to inhibit Akt by hydroxylation and binding of pVHL. As hypoxia inhibits PHD2, it activates and promotes tumor growth *via* Akt (34). PHD3 has been reported to interact with activating transcription factor-4, which has been shown to activate genes involved in redox balance, apoptosis, and general cell survival under conditions of compromised nutrition (35, 36). PHD3 has been associated with apoptosis *via* different mediators. For example, PHD3 induces a neuronal regulator kinesin family member 1B β (KIF1β) that in turn induces apoptosis. For this interaction, PHD3 hydroxylation activity was required, but it is still not known whether there is direct hydroxylation of KIF1β (37). PHD3 has been found to hydroxylate and activate the human homolog of the *Caenorhabditis elegans* biological clock protein CLK-2 (HCLK2), which is an important player in DNA damage response. This was required for the activation of the ATR/CHK1/p53 pathway, so that inhibition of PHD3 attenuated DNA damage and the consequent apoptosis (38). PHD3 also directly inactivates the β2-adrenergic receptor (β_2AR) by hydroxylating the intracellular domain, which leads to binding of the pVHL and subsequent degradation. β_2AR activation is also known to play a role in apoptosis (39, 40).

HIF AND PHDs IN TUMORIGENESIS: BAD COPS?

Extensive literature exists regarding the role of HIF and PHDs expressed by tumor cells. We will only touch on a few aspects here, and we recommend more in-depth reviews such as Rankin et al. (41) and Schito and Semenza (4) for more detailed information. Generally, HIF activation in cancer cells has been regarded

as tumor promoting. In solid tumors, this usually results from oxygen deprivation leading to hypoxia. However, HIF signaling can also be activated by other oncogenic or tumor suppressor pathways, but mutations directly affecting HIF activation are not common in cancer, with the exception of pVHL deficiency (42). As pVHL mediates the proteolysis of HIF-α subunits, inactivation of this protein leads to constitutively active HIF also in normoxic conditions. However, Von Hippel-Lindau mutations are responsible only for a limited set of cancers such as clear cell renal cancer (ccRCC), pheochromocytoma, and pancreatic neuroendocrine tumors. HIF-2α has been shown to be the driving force in ccRCC (43). HIF activation can also be controlled through PI3K/PTEN/AKT and RAS/RAF/MAPK signaling pathways. The former regulates the mTOR signaling that increases the translation of HIF protein, as described earlier. The latter has been reported to promote the HIF-coactivator p300 complex and subsequent transcription of target genes (42, 44, 45).

The metabolic components have also been shown to affect HIF stabilization through oxygen-independent regulation of PHD activity. The citric acid cycle (CAC) intermediate 2-OG is an absolute requirement for the enzymatic activity of PHD enzymes, whereas excess amount of other CAC metabolites fumarate, succinate, and oxaloacetate competitively inhibit PHDs (46). Inactivating mutations in enzymes processing fumarate and succinate leading to their accumulation, have been shown to induce HIF, and are associated with rare hereditary cancers, such as papillary renal cell carcinoma and paraganglioma (47–49). Mutations driving abnormal activation of isocitrate dehydrogenase (IDH) 3a were shown to decrease 2-OG amount causing HIF stabilization in cancer cells (50). Other types of IDH mutations lead to the production of 2-hydroxyglutarate (2-HG), which can competitively inhibit PHDs or even increase the activity depending on the 2-HG stereoisomerism (51, 52). However, the mechanisms for the PHD activation by 2-HG is debated (53). In triple negative breast cancer cells, the xCT glutamate-cystine antiporter has been shown to be inhibited by increased glutamate secretion. This in turn decreases the cellular cysteine level. Free cysteine prevents the oxidation of the specific cysteine residues in PHD2, but when the level decreases it leads to self-inactivation of PHD2 and subsequent HIF stabilization (54). Cancer cell-secreted lactate from glycolysis was shown to stabilize HIF in tumor-associated macrophages (TAMs) leading to induction in VEGF and arginase 1 (ARG1), markers associated with an M2 phenotype (55).

Multiple reports have showed that HIFs in cancer cells play a role in tumor progression. Inactivation of either HIF-1α or HIF-2α in *in vivo* breast cancer cell models decreases tumor growth and metastasis to the axillary lymph nodes (56), lung (57–61), and bone (62, 63). Hypoxia promotes cancer cell survival in multiple different ways and these have been reviewed in many papers (64, 65) These ways include the switch to anaerobic metabolism and neovascularization, which will be discussed further below. Hypoxia also promotes growth factor signaling, epithelial–mesenchymal transition, decrease in the apoptotic potential and evasion from the immune system. Cancer cells secrete growth factors to promote their own growth and survival as they generally also have membrane receptors for these factors, resulting in autocrine signaling. These growth factors also stimulate the surrounding stromal cells to change into a tumor promoting phenotype (65). HIF-1 has been shown to directly inhibit apoptosis by decreasing for instance the expression of the proapoptotic Bcl-2 family protein Bid (66) and to induce the expression of the apoptosis inhibitor survivin (67). In this way, cancer cells are protected from the harsh hypoxic environment leading also to a decrease in drug responsiveness (66). Hypoxia also modifies the cell surface proteins, which can shield the cells from immune system in many ways. For example, HIF-1 has been shown to directly upregulate programmed death-ligand 1 (PD-L1) (68) that suppresses T cell activation and CD47, which prevents phagocytosis by macrophages (69). HIF-1 induces a set of microRNAs that are small, non-coding RNA molecules known as hypoxamiRs (70). One of these is miR-210, which has been shown also to regulate cancer cell sensitivity toward cytotoxic T lymphocyte (CTL)-mediated lysis. miR-210 promotes cancer cell immune evasion without affecting cell surface proteins or CTL reactivity (71). In addition, HIF-induced miR-210 was shown also to promote angiogenesis. In cancer cells, miR-210 inhibits the expression and secretion of fibroblast growth factor receptor-like 1, which in turn was found to be a negative regulator of the angiogenesis (72).

In addition, hypoxia promotes cancer cell invasion and metastasis. For example, hypoxia enhances the motility and invasiveness of the cancer cells by promoting epithelial to mesenchymal transition. This happens by upregulating the expression of the transcription factors Snail1, Snail2, and Twist that downregulate expression of E-cadherin that is an important component of the adherens junctions. HIF also mediates this indirectly *via* Notch signaling (59, 73). Generally, CAFs are responsible for the remodeling of the ECM that assists in cancer cell invasion. However, numerous publications have proved that hypoxia induces cancer cells to secrete ECM remodeling enzymes that have essential roles in invasion, metastasis and premetastatic niche formation (60, 74–80). HIF-1 also mediates expression levels of angiopoietin-like 4 and L1 cell adhesion molecule. The former hampers vascular permeability and the latter cancer cell adhesion to the vasculature. Both are needed in intravasation and extravasation during the metastatic process (57).

Hypoxia also promotes the cancer stem cell phenotype. These cells are capable of unlimited cell division, differentiation to other cell types and, most importantly, can initiate cancer (59, 81). The hostile hypoxic microenvironment in the primary tumor primes the cancer cells to survive in the metastatic organ. There is some evidence that hypoxia plays a role in setting a dormancy phenotype by upregulating the main dormancy genes (NR2F1, DEC2, and p27) in breast cancer cells, which persist post-hypoxia helping the cancer cells to become therapy resistance (82).

Experiments have also been performed targeting PHDs that regulate HIF stability. Cancer cell-specific PHD2 haplodeficiency in the MMTV-PyMT breast cancer model led to increased HIF-1α and HIF-2α stabilization, but that did not have an effect on tumor growth or directly on the cancer cells' invasive behavior. However, reduced lung metastasis was seen (83). In the MDA-MB-231 breast cancer model, PHD2 inactivation in cancer cells leads also to attenuate tumor growth. This

resulted from decreased transforming growth factor β (TGF-β) processing leading to decreased expression of the extracellular protein osteopontin (SPP1), which has been associated with breast cancer malignancy (84). However, opposing results have been reported with the MCF-7 breast cancer model where PHD2 inactivation in cancer cells promoted tumor growth (84, 85) by upregulation of interleukin-8 (IL-8), VEGF, and the growth factor amphiregulin leading to increased vasculature formation (85). PHD2 inactivation led to HIF-1α stabilization and increased tumor growth in human colon carcinoma (HCT116), colorectal carcinoma (HT29 and RKO), and pancreatic carcinoma (SU.86.86) models. Generally, this was due to increased number of blood vessels. Further experiments with HCT116 cells revealed that the phenotype was not only HIF dependent. PHD2 was also shown to negatively regulate the transcription factor NF-κB. Hence, PHD2 inactivation led to increased secretion of angiogenic factors IL-8 and angiogenin (ANG) *via* NF-κB. These recruited bone marrow-derived cells (BMDCs) and promoted angiogenesis (86). PHD2 inactivation in murine osteosarcoma LM8, Lewis lung carcinoma (LLC), and B16BL6 melanoma cell lines also decreased tumor growth *in vivo*. The effect was claimed to be mainly HIF-independent since inactivation of HIF-1α led to increase in the tumor growth, which could be abolished when HIF-1α was inactivated together with PHD2. After more validation in the LM8 cell line, the PHD2 inactivation was shown to increase the number of vessels in the tumor but with decreased number of circulating cancer cells leading to reduced lung metastasis. LM8 cells were found to have increased expression of matrix metalloproteinases MMP2 and MT1MMP, which increased TGF-β signaling leading to decreased cancer cell proliferation (87, 88). PHD2 overexpression in the pancreatic cancer cells MIA PaCa-2 and PANC-1 decreased tumor growth by suppressing tumor vasculature in an HIF-dependent way resulting from decreases seen in VEGF and angiopoietin-1 (ANGPT1). However, this did not affect metastasis (89).

HIF prolyl hydroxylase 1 has been shown to play a role in cancer progression, but mainly in HIF-independent ways. PHD1 inactivation in ZR75-1, T47D, and MCF7 breast carcinoma cell lines decreased the tumor growth *in vivo* by HIF independently decreasing the cyclin D levels that are involved in cell cycling processes (90). Later PHD1 was shown to hydroxylate Forkhead box O3 (FOXO3), which hampers its interaction with the USP9x deubiquitinase leading to degradation. Decrease in FOXO3a decreases the cyclin D levels (91). Inhibition of PHD1 in HCT116 human colon carcinoma cells *in vitro* sensitized the cells for chemotherapy by decreasing p53 activation during the treatment and inhibited DNA repair increasing cell death (92).

HIF AND PHDs IN TUMOR STROMA: GOOD COPS?

It has become more evident that cancer progression is not only regulated by the cancer cells but also by the surrounding cancer stroma (93). In addition to the cancer cells, the TME includes different cells such as CAFs, endothelial cells (ECs), immune cells, growth factors, cytokines, and ECM (94). As described earlier, HIF activation in cancer cells has generally been regarded as a tumor-promoting factor, but opposing results have been shown with stromal cells. Global PHD2 haplodeficiency leading to increased stromal HIF-1α and HIF-2α stabilization did not have an effect on primary tumor growth in LLC and pancreatic carcinoma tumor models as well as in the MMTV-PyMT breast cancer metastasis model. Surprisingly, PHD2 haplodeficiency led to decreased lung metastasis, and this clearly demonstrated the importance of stromal hypoxic signaling on tumor progression (83, 95). Recent publications show that stromal factors together regulate the growth and metastatic capabilities of the cancer cells in a complex manner, but nevertheless revealing possible insights into new therapeutic targets.

HYPOXIC RESPONSE OF CAFs

Cancer cells are known to harness normal quiescent fibroblast to promote cancer progression and one way they achieve this is by secreting TGF-β. This activates the fibroblasts that become CAFs with increased capacity to contract and remodel the ECM (96). CAFs are the most abundant stromal cell type and are generally thought to promote cancer progression. Many coculture experiments with CAFs and cancer cells show increased tumor growth when compared with normal fibroblast together with cancer cells (97). CAFs can promote cancer cell invasion by secreting growth factors and cytokines or remodeling the ECM—either making tracks or aligning fibrils for cancer cells to travel along (98). When fibroblasts become CAFs, their gene expression changes, and one of the most commonly used marker for CAFs is α-smooth muscle actin (αSMA). In addition to αSMA, CAFs have been shown to express other markers such as fibroblast-specific protein 1 (FSP1), platelet-derived growth factor receptor (PDGFR), fibroblast activation protein, and periostin, for example. Recently, it has become clear that CAF populations are heterogeneous with diverse markers, which may give them specific functions during tumorigenesis (99).

HIF-1 has been shown to play a role in normal fibroblast and CAF behavior. One experiment showed HIF-1α as a tumor suppressor when its inactivation in FSP1-expressing cells in MMTV-PyMT transgenic mice led to enhanced tumor growth by reducing the tumor vascular density with less leaky vessels together with decreased infiltration of TAMs. The molecular mechanisms behind these results were not characterized. No difference was seen, however, in tumor growth in the same model with HIF-2α-deficient fibroblasts (100). Another experiment with *in vitro* data indicated that hypoxic CAFs are involved in formation of new blood vessels in a manner requiring both HIF-1α and G-protein estrogen receptor to upregulate VEGF in hypoxia (101). HIF-1α has been shown to work as a tumor promoting factor as well. Experiments using the active form of HIF-1α in human skin fibroblast showed increased tumor growth when co-injected together with MDA-MB-231 cells, whereas HIF-2α did not have an effect. It was suggested that HIF-1α activation led to autophagy and aerobic glycolysis, which would produce nutrients to surrounding cancer cells promoting their growth (102, 103). PHD2 modulation in CAFs also affects tumor progression, but

mainly *via* HIF. Hypoxia, PHD2 depletion, or PHD2 haplodeficiency was shown to inactivate CAFs by reducing their contraction and ECM remodeling capabilities (83, 104). Yes-associated protein 1 oncoprotein has been demonstrated to be important for CAF activation. It mediates the contraction *via* myosin light chain (MLC), which in turn regulates the contractile actomyosin function (105). Hypoxia and dimethyloxalylglycine (DMOG) decreased the levels of the phosphorylated MLC in CAFs (104). CAF inactivation was further demonstrated *in vivo* when CAFs with either PHD2 haplodeficiency or shRNA inactivation were orthotopically transplanted together with breast cancer cells leading to decreased lung metastases but without any changes in primary tumor growth (83, 104). Also, systemic PHD inhibition with DMOG decreased lung and liver metastases in a 4T1 breast cancer model. DMOG treatment also reduced the stiffness and αSMA levels within the tumors without affecting the number of αSMA positive cells (104). However, PHD2 haplodeficiency in platelet-derived growth factor receptor α (BDGFRα)-positive CAFs in MMTV-PyMT transgenic mice did not have an effect on the lung metastases (83). As a side note, targeting only one CAF marker might not ensure wide inactivation in a heterogeneous CAF population (99).

HYPOXIC RESPONSE OF THE TUMOR VASCULATURE

The tumor vasculature is essential in supplying nutrients and oxygen to the fast dividing cancer cells but becomes quickly inadequate to meet the need of a fast expanding tumor. Oxygen deprivation in the tumor causes HIF stabilization and upregulation of proangiogenic factors both from cancer cells and tumor stromal cells to promote the vessel formation needed for tumor progression and metastasis (106). ECM proteins have also been shown to be involved in angiogenesis. Hypoxia-inducible LOX promotes the expression of VEGF in cancer cells *via* PDGFRβ-mediated Akt activation (107). Tumor vasculature, however, has been found to be abnormal with morphological changes, leakiness, and aberrant pericytes, which result in poorly perfused vessels that are not able to rescue the hypoxic condition. This results from excessively secreted pro- and antiangiogenic factors leading to disorganized vessel formation. This can hinder, for example, the delivery of anticancer drugs and also benefit cancer cells during their extravasation process (108).

Inactivation of HIF-1α in Tie2 positive ECs lead to decreased tumor growth in a subcutaneous LLC model, caused by reduced density of tumor vessels. ECs had decreased proliferation and migration under hypoxia as well as decreased expression levels of VEGF and VEGF receptor-1 (VEGFR-1). This demonstrated the importance of EC autocrine expression of these factors during vessel formation (109). Similar results were obtained with the MMTV-PyMT breast cancer model and the LLC model. HIF-1α inactivation in Tie2-positive ECs drastically decreased lung metastasis, but only had moderate effect on primary tumor growth. In addition, HIF-1α inactivation decreased the expression of inducible nitric oxide synthase (iNOS), further decreasing the amount of nitric oxide (NO) and leading to less permeable ECs for cancer cell migration. HIF-2α inactivation in ECs on the other hand led to decreased levels of arginase 1 (ARG1), which in turn increased the NO levels making the EC layer more accessible for cancer cells. Supporting this finding, *in vivo* tail vein injection of LLC cells showed more cancer cell colonization in the lung of HIF-2α EC null mice (110). However, HIF-2α inactivation from vascular endothelial (VE)-cadherin positive ECs decreased tumor growth in LLC and B16F1 models, as well as in carcinogen-induced skin epithelial tumors. HIF-2α inactivation was shown to increase the number of vessels and their branching but decreasing the number of mature functional tumor vessels. This resulted from disrupted delta-like ligand 4/Notch (DII4/Notch) signaling leading to decreased expression of angiopoietin-2 (111, 112). These results show different roles for HIF-1 and HIF-2 in angiogenesis. Mice with PHD2 haplodeficiency in Tie2-positive ECs did not have an effect on the primary tumor growth, but the amount of liver metastases in a pancreatic cancer model was reduced. This resulted from HIF-2-induced expression of sVEGFR-1 and VE cadherin that normalized ECs with less permeable vessels leading to a less hypoxic tumor without any changes in the actual number of vessels (83, 95).

Pericytes are mural cells that cover the ECs and provide vascular stability and are known to signal to each other during angiogenesis (108). Pericyte depletion was shown to disrupt primary tumor growth by decreasing the microvessel density and increasing hypoxia, but this, however, led to increased metastasis showing their importance in tumor vasculature (113). Later, the increased hypoxia was shown to induce Ang-2 expression within the tumor which resulted in vascular instability and lung metastasis. This could be restored by inhibiting Ang-2 signaling (114).

HYPOXIC RESPONSE OF IMMUNE CELLS

The microenvironment of solid tumors also contains tumor infiltrating immune cells. These include lymphoid cells and myeloid cells, such as T- and B-cells and natural killer cells, TAMs, myeloid-derived suppressor cells (MDSCs), and neutrophils, which all have both pro- and antitumorigenic effects. Hypoxia has been shown to alter immune resistance and suppression, which helps tumor cells to survive immune surveillance (2). TAMs are the most common immune cells found within the tumor and are generally associated with tumor progression and also have been shown to promote cancer cell invasion and angiogenesis (115). Hypoxic tumor regions have been shown to recruit TAMs *via* VEGF and semaphoring 3A expression and once in a low oxygen condition their gene expression profile changes to a more cancer promoting phenotype (116, 117). Macrophages are known to have plasticity, and their phenotype can change based on external cues. Once they get activated they can have tumor suppressing M1 or tumor promoting M2 polarization. Stimulation driving the M1 phenotype also activates HIF-1 which upregulates iNOS levels and drives NO synthesis. During M2 polarization HIF-2 is induced leading to an increase in arginase-1, which in turn decreases the NO synthesis (118). *In vitro* assays suggest that depletion of HIF-1α in TAMs induces more M2 phenotype (119). By using PHD2-haplodeficient mice, which ensured less hypoxic tumors, Laoui et al. showed, however, that the oxygenation state in

the tumor did not significantly affect the differentiation of TAMs into M1 or M2 like (120). They saw changes in hypoxia-regulated genes in M2 like TAMs, but not in M1 like TAMs. Overall the results suggest that hypoxia itself does not influence the activation of the TAMs into M1 nor M2 (120).

HIF-1α- and HIF-2α-expressing macrophages have been shown to promote tumor progression. Inactivation of HIF-1α from lysozyme 2 (LysM)-positive neutrophils and macrophages in MMTV-PyMT mice led to hindered tumor progression and smaller tumors. This was shown to result from an increased number of cytotoxic T-cells (121). Mice lacking HIF-2α from LysM-positive macrophages in inducible hepatocellular carcinoma and colitis-associated cancer had reduced number of TAMs in tumor regions which associated with a delayed tumor progression tendency (122). However, opposing results have been shown with PHD inactivation. One experiment showed that PHD2 inactivation in macrophages together with $CD4^+$ T-cells decreased the tumor progression by overall downregulation of protumoral and antitumoral cytokines eventually leading to increased tumor cell death in an LLC model, and this was in part because of HIF-1α stabilization (123). Mice with a melanoma tumor model were treated with granulocyte–macrophage colony-stimulating factor together with systemic inhibition of PHD3 by AKB-6899 leading to decreased tumor size and lung metastasis. This resulted from HIF-2α stabilization in TAMs, which increased the expression of VEGF sequestering the soluble VEGFR-1 which decreased the vasculature within the tumor (124).

Hypoxia and HIF-1α stabilization regulates the MDSCs within the tumor enhancing their role as T-cell suppressors (125). Tumor-infiltrating MDSCs, as well as macrophages and tumor cells, were shown to have increased levels of the cell surface protein PD-L1, which was shown to be an HIF-1α target and upregulated by hypoxia. PD-L1 upregulation in MDSCs suppressed T-cell activation, whereas PD-L1 blockade under hypoxia enhanced MDSC-mediated T-cell proliferation and function and was associated with decreased interleukin-6 and interleukin-10 expression (68). Hypoxia has been shown to decrease the expression of the cell surface protein major histocompatibility complex class I *in vivo* and *in vitro* in renal cell carcinoma cells providing them a mechanism to evade immune surveillance by cytotoxic T-cells (126).

HYPOXIC RESPONSE OF THE ECM

In addition to the different cell types within the TME, the ECM composition and structure also contributes to tumor progression (94). The ECM does not only provide an architectural scaffold defining tissue boundaries but it also regulates cellular behavior, such as cell adhesion and migration by mechanical and biochemical cues. The ECM can bind and store growth factors or directly interact with receptors on the cell surfaces. The ECM is a dynamic meshwork of proteins that is constantly being remodeled and it is rich in proteoglycans and fibrillar collagens. ECM proteins have also been shown to directly induce cancer cell proliferation and metastasis. LOX is upregulated by hypoxia, and in a colorectal cancer model, LOX induced cancer progression *via* activation of SRC signaling (127). The ECM is mainly secreted by CAFs as it is one of the main functions of fibroblast to maintain the homeostasis of the ECM. CAFs, however, have abnormal ECM regulation activity, and they have been shown to secrete more ECM proteins than normal fibroblasts (98). In addition to CAFs, cancer cells are known to play a part in ECM remodeling (128). A high collagen content together with a stiff ECM is generally associated with malignant metastatic cancers (75, 129). The biosynthesis of the fibrillar collagens requires posttranslational modifications that take place both inside and outside the cell with the help of collagen modifying enzymes, many of which are known to be regulated by HIF (130). Collagen modifying enzymes have been shown to have important roles in cancer progression. Inactivation of collagen prolyl 4-hydroxylase P4HA1 or P4HA2 in MDA-MB-123 breast cancer cells decreased collagen deposition and attenuated tumor growth and resulted in as much as 99% decrease in lung metastasis (79). Inactivation of lysyl hydroxylase PLOD2 in the same cancer model decreased the metastasis to lungs resulting from inefficient formation of collagen fibers and decreased tumor stiffness (78). Inhibiting LOX and lysyl oxidase-like 2 (LOXL2) and LOXL4 decreased metastasis in several cancer cell types (60, 74, 76). LOX, LOXL2, and LOXL4 deficiencies decreased collagen cross-linking and stiffness which was also needed to recruit $CD11b^+$ BMDCs into the premetastatic niches to promote tumor cell colonization (60, 77, 80). LOXL2 in turn upregulated the expression of matrix remodeling enzymes such as tissue inhibitor of metalloproteinase-1 and matrix metalloproteinase-9 (MMP9) (76). LOXL2 also activated CAFs *via* integrin β3 and promoted their collagen contraction, a phenomenon known to increase matrix stiffness (131).

Cancer-associated fibroblasts are able to mechanically remodel the ECM by active contraction resulting in matrix stiffening (105). CAFs with PHD2 haplodeficiency were less active than wild-type CAFs which was demonstrated by decreased expression levels of markers associated with CAFs (αSMA, FSP1, and PDGFRα) and decreased contraction capabilities (83). PHD2 haplodeficiency in CAFs also decreased matrix production, and this was accompanied by decreased expression of collagen modifying enzymes LOX, P4HA1, P4HA2, PLOD2 as well as matrix metalloproteinases MMP2 and MMP9. This is partly surprising, since hypoxic fibroblast and cancer cells generally upregulate the expression of the same genes (132). Similar results were seen with human head and neck carcinoma-associated fibroblasts and human vulvar carcinoma-associated fibroblasts. PHD2 inactivation or 1% oxygen decreased αSMA expression, and they had decreased contraction capabilities (104).

HYPOXIC RESPONSE OF CANCER METABOLISM

One of the most common characteristics for cancer cells is rapid proliferation, which is accompanied by high demands of energy. In normoxia, cells generally produce energy as ATP by mitochondrial oxidative phosphorylation, which is an O_2-dependent pathway. As tumors grow, cells face the limitation of decreased

oxygen concentration and switch to inefficient glycolysis to ensure ATP production (46). HIF-1 has been shown to be an important regulator in the metabolic switch as it induces the expression of genes that adjust the cellular metabolism away from oxidative phosphorylation toward increased glycolysis. This mechanism is not just to ensure the ATP production under hypoxia but also to prevent excessive formation of reactive oxygen species (ROS). Most importantly HIF-1 induces pyruvate dehydrogenase kinase 1 that inhibits the activity of the pyruvate dehydrogenase complex (PDC). PDC regulates the first step needed in oxidative phosphorylation where pyruvate is converted to acetyl coenzyme A (133). HIF-1α also upregulates lactate dehydrogenase A and monocarboxylate transporter 4 which promotes conversion of pyruvate to lactate (end product of glycolysis) and transports it out of the cell, respectively (134, 135). HIF activation also upregulates the expression of glucose transporters GLUT1 and GLUT3, which are responsible for the glucose import into the cells (136, 137). Lactate from glycolysis is considered as a major reason for the tumor acidification. Hypoxia upregulates carbonic anhydrase IX, which regulates the tumor pH and has an important role in the survival of tumor cells in hypoxic regions of tumors and metastasis (138). In addition, HIF indirectly targets the metabolic genes that contribute to the glycolytic shift by upregulating miR-210. miR-210 in turn inhibits multiple targets such as iron-sulfur cluster assembly proteins (ISCU), cytochrome *c* oxidase assembly protein (COX10), NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4 (NDUFA4), and succinate dehydrogenase complex subunit D (SDHD), all of which play a role in mitochondrial function (139–141). PHD3 was in turn shown to induce the activity of PDC HIF independently by binding to the complex. PHD3 deficient cells were seen to have clear decreased PDC activity, which promoted survival in prolonged hypoxia together with a lower amount of ROS (142). HIF also suppresses mitochondrial function by downregulating multiple components in the electron transport chain and also by direct mitochondrial autophagy mediated by increased BCL2 interacting protein 3 (BNIP3) levels. In this way, hypoxia also decreases the levels of ROS, which are harmful in increased quantities (143). There are reports stating that mitochondrial ROS itself also regulates HIF, but there is also many opposing results [reviewed in Ref. (144)]. Increased ROS levels have been shown to promote HIF stabilization during hypoxia, by further inhibiting PHD enzymes. Contradicting results claim it is not ROS regulating HIF stabilization, but rather the mitochondria, which monitor the intracellular oxygen availability. Under normoxic conditions ROS are able to stabilize HIF, but the exact mechanism is not clear. It has been suggested to be *via* inhibition of PHDs or pVHL, but another signaling pathway has been proposed. HIF-1α gene expression was shown to be upregulated in oral squamous cell carcinoma and MCF-7 breast cancer cells by ROS *via* induced ERK and P13K/AKT signaling (145, 146). Overall, HIF-regulated glucose metabolism supplies sufficient glucose import for less efficient glycolysis and removes the excess end products out of the cell. It defends the cells against increased

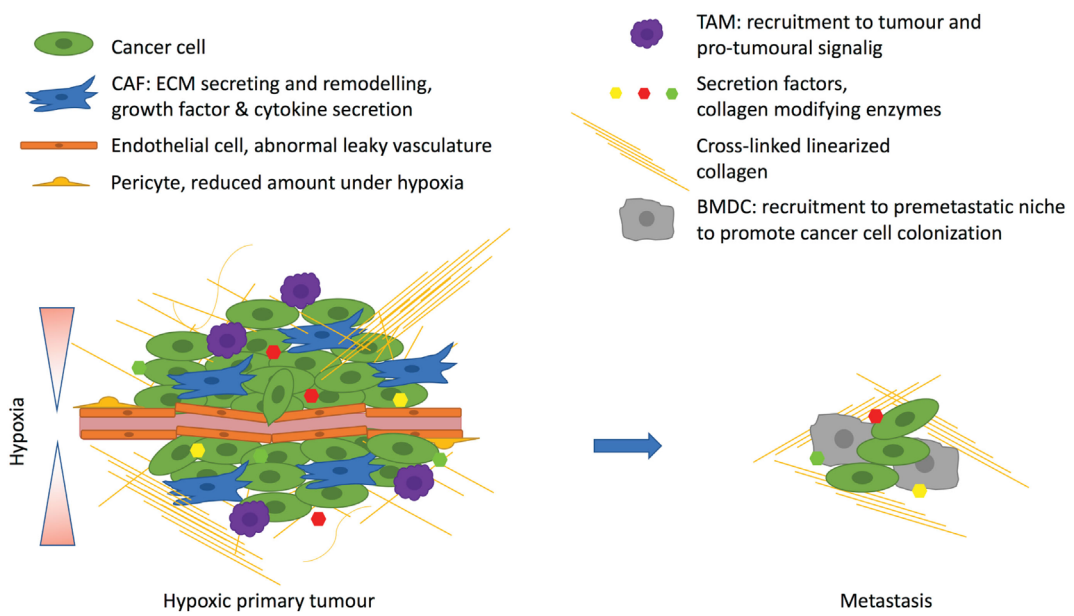


FIGURE 2 | Hypoxia affects the tumor microenvironment. Hypoxia arises due to rapidly proliferating cancer cells with inadequate vasculature. The excessive angiogenic signaling promotes formation of abnormal leaky vasculature with a decreased amount of pericytes. Hypoxia promotes tumor-associated macrophage (TAM) infiltration, which support tumor growth. Hypoxic cancer cells corrupt fibroblasts by secreting, for example, transforming growth factor β, which turns fibroblast into cancer-associated fibroblasts (CAFs) to drive the cancer progression. CAFs promote desomplasia and together with cancer cells they secrete extracellular matrix (ECM) components such as collagens. One important secreted factor is the hypoxia-inducible enzyme lysyl oxidase, which cross-links fibrillar collagen at the primary tumor and prepares the premetastatic niche for the recruitment of bone marrow derived cells (BMDCs) that are needed for cancer cell colonization at the metastatic site.

ROS production, which is generated in the oxidative phosphorylation pathway under too low oxygen concentration and gives the cancer cells protection to proliferate and grow under hypoxic conditions (147).

Within the solid tumor there are regions that are not under hypoxia, but still the cancer cells have a tendency to switch to inefficient glycolysis even in presence of oxygen. However, the metabolic state in the different cells within the tumor is heterogeneous and there has been evidence of supplying metabolites between cells (148). When fibroblasts become CAFs, they have been shown to decrease the expression of isocitrate dehydrogenase 3α. This leads to a decreased amount of effective 2-oxoglutarate, which is needed as a co-substrate in the hydroxylation reaction, and increasing levels of succinate and fumarate. This results to PHD inhibition, which stabilizes HIF-1α and promotes glycolysis in the CAFs. Metabolites from the normoxic glycolysis in CAFs was hypothesized to feed the surrounding cancer cells (149).

CONCLUSION AND FUTURE PERSPECTIVES

Hypoxia is a condition that plays a role in normal physiology, such as during embryonic development, but it is more commonly associated with pathological states. Oxygen deprivation is a common feature of all solid tumors and affects all components of the TME (**Figure 2**). Hypoxia has clear effects on both cancer cells and surrounding stromal cells, but it does not promote tumor progression in every case. Different cell types seem to be differentially regulated by the hypoxic environment. In cancer cells, HIF stabilization generally promotes tumor progression. Inactivation of HIF-1 or HIF-2 in cancer cells in mouse cancer models decreases the tumor growth and the formation of metastases in many ways, practically affecting all the hallmarks of cancer. When cancer cells are targeted with PHD2 inactivation, which enables HIF stabilization, the results are more variable. PHD enzymes do also have HIF-independent targets and functions that can complicate their role in cancer promotion. However, PHD2 haplodeficiency in ECs leads to less tumor hypoxia resulting from HIF-2 activation in the ECs themselves stimulating EC intrinsic proangiogenic signaling leading to EC normalization. Global haplodeficient inactivation of PHD2 or treatment with a PHD inhibitor does not have an effect on primary tumor growth, but it rather decreases metastasis. Targeting PHD2 in CAFs leads to their deactivation and reduces their capabilities to promote cancer cell invasion and metastasis both *in vitro* and *in vivo*. These findings suggest that global targeting of PHD and HIF activities is sufficient to reprogramme the TME to suppress tumor progression. However, we are only beginning to understand how hypoxia, HIFs and PHDs alter the TME, and further research is required to gain insight. Thus, while hypoxia research has been performed for decades, recent data suggests that there is much more to learn, and that this may have important clinical implications regarding the use of agents that target hypoxia pathways such as HIF or PHD inhibitors.

AUTHOR CONTRIBUTIONS

The review was written by AL and edited by JTE.

ACKNOWLEDGMENTS

We would like to thank Kamilla Westarp Zornhagen for the helpful comments for the manuscript. We thank the KBVU grant (R146-A9250) from the Danish Cancer Society that supports AL and the Novo Nordisk Foundation for the Hallas Møller Stipendum that supports JTE.

REFERENCES

1. Vaupel P, Mayer A. Hypoxia in cancer: significance and impact on clinical outcome. *Cancer Metastasis Rev* (2007) 26:225–39. doi:10.1007/s10555-007-9055-1
2. LaGory EL, Giaccia AJ. The ever-expanding role of HIF in tumour and stromal biology. *Nat Cell Biol* (2016) 18:356–65. doi:10.1038/ncb3330
3. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell* (2012) 21:309–22. doi:10.1016/j.ccr.2012.02.022
4. Schito L, Semenza GL. Hypoxia-inducible factors: master regulators of cancer progression. *Trends Cancer* (2016) 2:758–70. doi:10.1016/j.trecan.2016.10.016
5. Span PN, Bussink J. Biology of hypoxia. *Semin Nucl Med* (2015) 45:101–9. doi:10.1053/j.semnuclmed.2014.10.002
6. Wouters BG, Koritzinsky M. Hypoxia signalling through mTOR and the unfolded protein response in cancer. *Nat Rev Cancer* (2008) 8:851–64. doi:10.1038/nrc2501
7. Giampietri C, Petrungaro S, Conti S, Facchiano A, Filippini A, Ziparo E. Cancer microenvironment and endoplasmic reticulum stress response. *Mediators Inflamm* (2015) 2015:417281. doi:10.1155/2015/417281
8. Pereira ER, Frudd K, Awad W, Hendershot LM. Endoplasmic reticulum (ER) stress and hypoxia response pathways interact to potentiate hypoxia-inducible factor 1 (HIF-1) transcriptional activity on targets like vascular endothelial growth factor (VEGF). *J Biol Chem* (2014) 289:3352–64. doi:10.1074/jbc.M113.507194
9. Vanacker H, Vetters J, Moudombi L, Caux C, Janssens S, Michallet M-C. Emerging role of the unfolded protein response in tumor immunosurveillance. *Trends Cancer* (2017) 3:491–505. doi:10.1016/j.trecan.2017.05.005
10. Xu K, Liu P, Wei W. mTOR signaling in tumorigenesis. *Biochim Biophys Acta* (2014) 1846:638–54. doi:10.1016/j.bbcan.2014.10.007
11. Heberle AM, Prentzell MT, van Eunen K, Bakker BM, Grellscheid SN, Thedieck K. Molecular mechanisms of mTOR regulation by stress. *Mol Cell Oncol* (2015) 2:e970489. doi:10.4161/23723548.2014.970489
12. Arsham AM, Howell JJ, Simon MC. A novel hypoxia-inducible factor-independent hypoxic response regulating mammalian target of rapamycin and its targets. *J Biol Chem* (2003) 278:29655–60. doi:10.1074/jbc.M212770200
13. Shoshani T, Faerman A, Mett I, Zelin E, Tenne T, Gorodin S, et al. Identification of a novel hypoxia-inducible factor 1-responsive gene, RTP801, involved in apoptosis. *Mol Cell Biol* (2002) 22:2283–93. doi:10.1128/MCB.22.7.2283-2293.2002
14. Wolff NC, Vega-Rubin-de-Celis S, Xie X-J, Castrillon DH, Kabbani W, Brugarolas J. Cell-type-dependent regulation of mTORC1 by REDD1 and the tumor suppressors TSC1/TSC2 and LKB1 in response to hypoxia. *Mol Cell Biol* (2011) 31:1870–84. doi:10.1128/MCB.01393-10
15. Gao Y, Xiao Q, Ma H, Li L, Liu J, Feng Y, et al. LKB1 inhibits lung cancer progression through lysyl oxidase and extracellular matrix remodeling. *Proc Natl Acad Sci U S A* (2010) 107:18892–7. doi:10.1073/pnas.1004952107
16. Dodd KM, Yang J, Shen MH, Sampson JR, Tee AR. mTORC1 drives HIF-1α and VEGF-A signalling via multiple mechanisms involving 4E-BP1, S6K1 and STAT3. *Oncogene* (2015) 34:2239–50. doi:10.1038/onc.2014.164

17. Al-Ansari MM, Hendrayani S-F, Tulbah A, Al-Tweigeri T, Shehata AI, Aboussekhra A. p16INK4A represses breast stromal fibroblasts migration/invasion and their VEGF-A-dependent promotion of angiogenesis through Akt inhibition. *Neoplasia* (2012) 14:1269–77. doi:10.1593/neo.121632
18. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, et al. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science* (2001) 292:464–8. doi:10.1126/science.1059817
19. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, et al. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science* (2001) 292:468–72. doi:10.1126/science.1059796
20. Masson N, Willam C, Maxwell PH, Pugh CW, Ratcliffe PJ. Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. *EMBO J* (2001) 20:5197–206. doi:10.1093/emboj/20.18.5197
21. Yu F, White SB, Zhao Q, Lee FS. HIF-1alpha binding to VHL is regulated by stimulus-sensitive proline hydroxylation. *Proc Natl Acad Sci U S A* (2001) 98:9630–5. doi:10.1073/pnas.181341498
22. Hu C-J, Sataur A, Wang L, Chen H, Simon MC. The N-terminal transactivation domain confers target gene specificity of hypoxia-inducible factors HIF-1alpha and HIF-2alpha. *Mol Biol Cell* (2007) 18:4528–42. doi:10.1091/mbc.e06-05-0419
23. Heikkilä M, Pasanen A, Kivirikko KI, Myllyharju J. Roles of the human hypoxia-inducible factor (HIF)-3α variants in the hypoxia response. *Cell Mol Life Sci* (2011) 68:3885–901. doi:10.1007/s00018-011-0679-5
24. Makino Y, Uenishi R, Okamoto K, Isoe T, Hosono O, Tanaka H, et al. Transcriptional up-regulation of inhibitory PAS domain protein gene expression by hypoxia-inducible factor 1 (HIF-1): a negative feedback regulatory circuit in HIF-1-mediated signaling in hypoxic cells. *J Biol Chem* (2007) 282:14073–82. doi:10.1074/jbc.M700732200
25. Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* (2001) 294:1337–40. doi:10.1126/science.1066373
26. Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, et al. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* (2001) 107:43–54. doi:10.1016/S0092-8674(01)00507-4
27. Dayan F, Roux D, Brahimi-Horn MC, Pouyssegur J, Mazure NM. The oxygen sensor factor-inhibiting hypoxia-inducible factor-1 controls expression of distinct genes through the bifunctional transcriptional character of hypoxia-inducible factor-1alpha. *Cancer Res* (2006) 66:3688–98. doi:10.1158/0008-5472.CAN-05-4564
28. Hirsilä M, Koivunen P, Günzler V, Kivirikko KI, Myllyharju J. Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. *J Biol Chem* (2003) 278:30772–80. doi:10.1074/jbc.M304982200
29. Berra E, Benizri E, Ginouvès A, Volmat V, Roux D, Pouysségur J. HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia. *EMBO J* (2003) 22:4082–90. doi:10.1093/emboj/cdg392
30. Takeda K, Ho VC, Takeda H, Duan L-J, Nagy A, Fong G-H. Placental but not heart defects are associated with elevated hypoxia-inducible factor alpha levels in mice lacking prolyl hydroxylase domain protein 2. *Mol Cell Biol* (2006) 26:8336–46. doi:10.1128/MCB.00425-06
31. Appelhoff RJ, Tian Y-M, Raval RR, Turley H, Harris AL, Pugh CW, et al. Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. *J Biol Chem* (2004) 279:38458–65. doi:10.1074/jbc.M406026200
32. Aprelikova O, Chandramouli GVR, Wood M, Vasselli JR, Riss J, Maranchie JK, et al. Regulation of HIF prolyl hydroxylases by hypoxia-inducible factors. *J Cell Biochem* (2004) 92:491–501. doi:10.1002/jcb.20067
33. To KKW, Huang LE. Suppression of hypoxia-inducible factor 1alpha (HIF-1alpha) transcriptional activity by the HIF prolyl hydroxylase EGLN1. *J Biol Chem* (2005) 280:38102–7. doi:10.1074/jbc.M504342200
34. Guo J, Chakraborty AA, Liu P, Gan W, Zheng X, Inuzuka H, et al. pVHL suppresses kinase activity of Akt in a proline-hydroxylation-dependent manner. *Science* (2016) 353:929–32. doi:10.1126/science.aad5755
35. Hiwatashi Y, Kanno K, Takasaki C, Goryo K, Sato T, Torii S, et al. PHD1 interacts with ATF4 and negatively regulates its transcriptional activity without prolyl hydroxylation. *Exp Cell Res* (2011) 317:2789–99. doi:10.1016/j.yexcr.2011.09.005
36. Köditz J, Nesper J, Wottawa M, Stiehl DP, Camenisch G, Franke C, et al. Oxygen-dependent ATF-4 stability is mediated by the PHD3 oxygen sensor. *Blood* (2007) 110:3610–7. doi:10.1182/blood-2007-06-094441
37. Schlisio S, Kenchappa RS, Vredeveld LCW, George RE, Stewart R, Greulich H, et al. The kinesin KIF1Bbeta acts downstream from EglN3 to induce apoptosis and is a potential 1p36 tumor suppressor. *Genes Dev* (2008) 22:884–93. doi:10.1101/gad.1648608
38. Xie L, Pi X, Mishra A, Fong G, Peng J, Patterson C. PHD3-dependent hydroxylation of HCLK2 promotes the DNA damage response. *J Clin Invest* (2012) 122:2827–36. doi:10.1172/JCI62374
39. Xie L, Xiao K, Whalen EJ, Forrester MT, Freeman RS, Fong G, et al. Oxygen-regulated beta(2)-adrenergic receptor hydroxylation by EGLN3 and ubiquitylation by pVHL. *Sci Signal* (2009) 2:ra33. doi:10.1126/scisignal.2000444
40. Yang M, Su H, Soga T, Kranc KR, Pollard PJ. Prolyl hydroxylase domain enzymes: important regulators of cancer metabolism. *Hypoxia (Auckl)* (2014) 2:127–42. doi:10.2147/HP.S47968
41. Rankin EB, Nam J-M, Giaccia AJ. Hypoxia: signaling the metastatic cascade. *Trends Cancer* (2016) 2:295–304. doi:10.1016/j.trecan.2016.05.006
42. Masson N, Ratcliffe PJ. Hypoxia signaling pathways in cancer metabolism: the importance of co-selecting interconnected physiological pathways. *Cancer Metab* (2014) 2:3. doi:10.1186/2049-3002-2-3
43. Tarade D, Ohh M. The HIF and other quandaries in VHL disease. *Oncogene* (2018) 37:139–47. doi:10.1038/onc.2017.338
44. Pore N, Jiang Z, Shu H-K, Bernhard E, Kao GD, Maity A. Akt1 activation can augment hypoxia-inducible factor-1alpha expression by increasing protein translation through a mammalian target of rapamycin-independent pathway. *Mol Cancer Res* (2006) 4:471–9. doi:10.1158/1541-7786.MCR-05-0234
45. Sang N, Stiehl DP, Bohensky J, Leshchinsky I, Srinivas V, Caro J. MAPK signaling up-regulates the activity of hypoxia-inducible factors by its effects on p300. *J Biol Chem* (2003) 278:14013–9. doi:10.1074/jbc.M209702200
46. Nguyen TL, Durán RV. Prolyl hydroxylase domain enzymes and their role in cell signaling and cancer metabolism. *Int J Biochem Cell Biol* (2016) 80:71–80. doi:10.1016/j.biocel.2016.09.026
47. Pollard PJ, Brière JJ, Alam NA, Barwell J, Barclay E, Wortham NC, et al. Accumulation of Krebs cycle intermediates and over-expression of HIF1alpha in tumours which result from germline FH and SDH mutations. *Hum Mol Genet* (2005) 14:2231–9. doi:10.1093/hmg/ddi227
48. Isaacs JS, Jung YJ, Mole DR, Lee S, Torres-Cabala C, Chung Y-L, et al. HIF overexpression correlates with biallelic loss of fumarate hydratase in renal cancer: novel role of fumarate in regulation of HIF stability. *Cancer Cell* (2005) 8:143–53. doi:10.1016/j.ccr.2005.06.017
49. Selak MA, Armour SM, MacKenzie ED, Boulahbel H, Watson DG, Mansfield KD, et al. Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. *Cancer Cell* (2005) 7:77–85. doi:10.1016/j.ccr.2004.11.022
50. Zeng L, Morinibu A, Kobayashi M, Zhu Y, Wang X, Goto Y, et al. Aberrant IDH3α expression promotes malignant tumor growth by inducing HIF-1-mediated metabolic reprogramming and angiogenesis. *Oncogene* (2015) 34:4758–66. doi:10.1038/onc.2014.411
51. Koivunen P, Lee S, Duncan CG, Lopez G, Lu G, Ramkissoon S, et al. Transformation by the (R)-enantiomer of 2-hydroxyglutarate linked to EGLN activation. *Nature* (2012) 483:484–8. doi:10.1038/nature10898
52. Burr SP, Costa ASH, Grice GL, Timms RT, Lobb IT, Freisinger P, et al. Mitochondrial protein lipoylation and the 2-oxoglutarate dehydrogenase complex controls HIF1α stability in aerobic conditions. *Cell Metab* (2016) 24:740–52. doi:10.1016/j.cmet.2016.09.015
53. Tarhonskaya H, Rydzik AM, Leung IKH, Loik ND, Chan MC, Kawamura A, et al. Non-enzymatic chemistry enables 2-hydroxyglutarate-mediated activation of 2-oxoglutarate oxygenases. *Nat Commun* (2014) 5:3423. doi:10.1038/ncomms4423
54. Briggs KJ, Koivunen P, Cao S, Backus KM, Olenchock BA, Patel H, et al. Paracrine induction of HIF by glutamate in breast cancer: egln1 senses cysteine. *Cell* (2016) 166:126–39. doi:10.1016/j.cell.2016.05.042
55. Colegio OR, Chu N-Q, Szabo AL, Chu T, Rhebergen AM, Jairam V, et al. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature* (2014) 513:559–63. doi:10.1038/nature13490
56. Schito L, Rey S, Tafani M, Zhang H, Wong CC-L, Russo A, et al. Hypoxia-inducible factor 1-dependent expression of platelet-derived growth factor B

promotes lymphatic metastasis of hypoxic breast cancer cells. *Proc Natl Acad Sci U S A* (2012) 109:E2707–16. doi:10.1073/pnas.1214019109
57. Zhang H, Wong CCL, Wei H, Gilkes DM, Korangath P, Chaturvedi P, et al. HIF-1-dependent expression of angiopoietin-like 4 and L1CAM mediates vascular metastasis of hypoxic breast cancer cells to the lungs. *Oncogene* (2012) 31:1757–70. doi:10.1038/onc.2011.365
58. Liao D, Corle C, Seagroves TN, Johnson RS. Hypoxia-inducible factor-1alpha is a key regulator of metastasis in a transgenic model of cancer initiation and progression. *Cancer Res* (2007) 67:563–72. doi:10.1158/0008-5472.CAN-06-2701
59. Schwab LP, Peacock DL, Majumdar D, Ingels JF, Jensen LC, Smith KD, et al. Hypoxia-inducible factor 1α promotes primary tumor growth and tumor-initiating cell activity in breast cancer. *Breast Cancer Res* (2012) 14:R6. doi:10.1186/bcr3087
60. Wong CC-L, Gilkes DM, Zhang H, Chen J, Wei H, Chaturvedi P, et al. Hypoxia-inducible factor 1 is a master regulator of breast cancer metastatic niche formation. *Proc Natl Acad Sci U S A* (2011) 108:16369–74. doi:10.1073/pnas.1113483108
61. Wong CC-L, Zhang H, Gilkes DM, Chen J, Wei H, Chaturvedi P, et al. Inhibitors of hypoxia-inducible factor 1 block breast cancer metastatic niche formation and lung metastasis. *J Mol Med* (2012) 90:803–15. doi:10.1007/s00109-011-0855-y
62. Hiraga T, Kizaka-Kondoh S, Hirota K, Hiraoka M, Yoneda T. Hypoxia and hypoxia-inducible factor-1 expression enhance osteolytic bone metastases of breast cancer. *Cancer Res* (2007) 67:4157–63. doi:10.1158/0008-5472.CAN-06-2355
63. Dunn LK, Mohammad KS, Fournier PGJ, McKenna CR, Davis HW, Niewolna M, et al. Hypoxia and TGF-beta drive breast cancer bone metastases through parallel signaling pathways in tumor cells and the bone microenvironment. *PLoS One* (2009) 4:e6896. doi:10.1371/journal.pone.0006896
64. Keith B, Johnson RS, Simon MC. HIF1α and HIF2α: sibling rivalry in hypoxic tumour growth and progression. *Nat Rev Cancer* (2011) 12:9–22. doi:10.1038/nrc3183
65. Semenza GL. Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy. *Trends Pharmacol Sci* (2012) 33:207–14. doi:10.1016/j.tips.2012.01.005
66. Erler JT, Cawthorne CJ, Williams KJ, Koritzinsky M, Wouters BG, Wilson C, et al. Hypoxia-mediated down-regulation of Bid and Bax in tumors occurs via hypoxia-inducible factor 1-dependent and -independent mechanisms and contributes to drug resistance. *Mol Cell Biol* (2004) 24:2875–89. doi:10.1128/MCB.24.7.2875-2889.2004
67. Peng X-H, Karna P, Cao Z, Jiang B-H, Zhou M, Yang L. Cross-talk between epidermal growth factor receptor and hypoxia-inducible factor-1alpha signal pathways increases resistance to apoptosis by up-regulating survivin gene expression. *J Biol Chem* (2006) 281:25903–14. doi:10.1074/jbc.M603414200
68. Noman MZ, Desantis G, Janji B, Hasmim M, Karray S, Dessen P, et al. PD-L1 is a novel direct target of HIF-1α, and its blockade under hypoxia enhanced MDSC-mediated T cell activation. *J Exp Med* (2014) 211:781–90. doi:10.1084/jem.20131916
69. Zhang H, Lu H, Xiang L, Bullen JW, Zhang C, Samanta D, et al. HIF-1 regulates CD47 expression in breast cancer cells to promote evasion of phagocytosis and maintenance of cancer stem cells. *Proc Natl Acad Sci U S A* (2015) 112:E6215–23. doi:10.1073/pnas.1520032112
70. Nallamshetty S, Chan SY, Loscalzo J. Hypoxia: a master regulator of microRNA biogenesis and activity. *Free Radic Biol Med* (2013) 64:20–30. doi:10.1016/j.freeradbiomed.2013.05.022
71. Noman MZ, Buart S, Romero P, Ketari S, Janji B, Mari B, et al. Hypoxia-inducible miR-210 regulates the susceptibility of tumor cells to lysis by cytotoxic T cells. *Cancer Res* (2012) 72:4629–41. doi:10.1158/0008-5472.CAN-12-1383
72. Yang Y, Zhang J, Xia T, Li G, Tian T, Wang M, et al. MicroRNA-210 promotes cancer angiogenesis by targeting fibroblast growth factor receptor-like 1 in hepatocellular carcinoma. *Oncol Rep* (2016) 36:2553–62. doi:10.3892/or.2016.5129
73. Sahlgren C, Gustafsson MV, Jin S, Poellinger L, Lendahl U. Notch signaling mediates hypoxia-induced tumor cell migration and invasion. *Proc Natl Acad Sci U S A* (2008) 105:6392–7. doi:10.1073/pnas.0802047105
74. Erler JT, Bennewith KL, Nicolau M, Dornhöfer N, Kong C, Le Q-T, et al. Lysyl oxidase is essential for hypoxia-induced metastasis. *Nature* (2006) 440:1222–6. doi:10.1038/nature04695
75. Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell* (2009) 139:891–906. doi:10.1016/j.cell.2009.10.027
76. Barker HE, Chang J, Cox TR, Lang G, Bird D, Nicolau M, et al. LOXL2-mediated matrix remodeling in metastasis and mammary gland involution. *Cancer Res* (2011) 71:1561–72. doi:10.1158/0008-5472.CAN-10-2868
77. Cox TR, Bird D, Baker A-M, Barker HE, Ho MW-Y, Lang G, et al. LOX-mediated collagen crosslinking is responsible for fibrosis-enhanced metastasis. *Cancer Res* (2013) 73:1721–32. doi:10.1158/0008-5472.CAN-12-2233
78. Gilkes DM, Bajpai S, Wong CC, Chaturvedi P, Hubbi ME, Wirtz D, et al. Procollagen lysyl hydroxylase 2 is essential for hypoxia-induced breast cancer metastasis. *Mol Cancer Res* (2013) 11:456–66. doi:10.1158/1541-7786.MCR-12-0629
79. Gilkes DM, Chaturvedi P, Bajpai S, Wong CC, Wei H, Pitcairn S, et al. Collagen prolyl hydroxylases are essential for breast cancer metastasis. *Cancer Res* (2013) 73:3285–96. doi:10.1158/0008-5472.CAN-12-3963
80. Erler JT, Bennewith KL, Cox TR, Lang G, Bird D, Koong A, et al. Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. *Cancer Cell* (2009) 15:35–44. doi:10.1016/j.ccr.2008.11.012
81. Peng G, Liu Y. Hypoxia-inducible factors in cancer stem cells and inflammation. *Trends Pharmacol Sci* (2015) 36:374–83. doi:10.1016/j.tips.2015.03.003
82. Fluegen G, Avivar-Valderas A, Wang Y, Padgen MR, Williams JK, Nobre AR, et al. Phenotypic heterogeneity of disseminated tumour cells is preset by primary tumour hypoxic microenvironments. *Nat Cell Biol* (2017) 19:120–32. doi:10.1038/ncb3465
83. Kuchnio A, Moens S, Bruning U, Kuchnio K, Cruys B, Thienpont B, et al. The cancer cell oxygen sensor PHD2 promotes metastasis via activation of cancer-associated fibroblasts. *Cell Rep* (2015) 12:992–1005. doi:10.1016/j.celrep.2015.07.010
84. Wottawa M, Leisering P, von Ahlen M, Schnelle M, Vogel S, Malz C, et al. Knockdown of prolyl-4-hydroxylase domain 2 inhibits tumor growth of human breast cancer MDA-MB-231 cells by affecting TGF-β1 processing. *Int J Cancer* (2013) 132:2787–98. doi:10.1002/ijc.27982
85. Bordoli MR, Stiehl DP, Borsig L, Kristiansen G, Hausladen S, Schraml P, et al. Prolyl-4-hydroxylase PHD2- and hypoxia-inducible factor 2-dependent regulation of amphiregulin contributes to breast tumorigenesis. *Oncogene* (2011) 30:548–60. doi:10.1038/onc.2010.433
86. Chan DA, Kawahara TLA, Sutphin PD, Chang HY, Chi J-T, Giaccia AJ. Tumor vasculature is regulated by PHD2-mediated angiogenesis and bone marrow-derived cell recruitment. *Cancer Cell* (2009) 15:527–38. doi:10.1016/j.ccr.2009.04.010
87. Klotzsche-von Ameln A, Muschter A, Mamlouk S, Kalucka J, Prade I, Franke K, et al. Inhibition of HIF prolyl hydroxylase-2 blocks tumor growth in mice through the antiproliferative activity of TGFβ. *Cancer Res* (2011) 71:3306–16. doi:10.1158/0008-5472.CAN-10-3838
88. Klotzsche-von Ameln A, Muschter A, Heimesaat MM, Breier G, Wielockx B. HIF prolyl hydroxylase-2 inhibition diminishes tumor growth through matrix metalloproteinase-induced TGFβ activation. *Cancer Biol Ther* (2012) 13:216–23. doi:10.4161/cbt.13.4.18830
89. Su Y, Loos M, Giese N, Metzen E, Büchler MW, Friess H, et al. Prolyl hydroxylase-2 (PHD2) exerts tumor-suppressive activity in pancreatic cancer. *Cancer* (2012) 118:960–72. doi:10.1002/cncr.26344
90. Zhang Q, Gu J, Li L, Liu J, Luo B, Cheung H-W, et al. Control of cyclin D1 and breast tumorigenesis by the EglN2 prolyl hydroxylase. *Cancer Cell* (2009) 16:413–24. doi:10.1016/j.ccr.2009.09.029
91. Zheng X, Zhai B, Koivunen P, Shin SJ, Lu G, Liu J, et al. Prolyl hydroxylation by EglN2 destabilizes FOXO3a by blocking its interaction with the USP9x deubiquitinase. *Genes Dev* (2014) 28:1429–44. doi:10.1101/gad.242131.114
92. Deschoemaeker S, Di Conza G, Lilla S, Martín-Pérez R, Mennerich D, Boon L, et al. PHD1 regulates p53-mediated colorectal cancer chemoresistance. *EMBO Mol Med* (2015) 7:1350–65. doi:10.15252/emmm.201505492
93. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. *Nat Med* (2013) 19:1423–37. doi:10.1038/nm.3394

94. Wang M, Zhao J, Zhang L, Wei F, Lian Y, Wu Y, et al. Role of tumor microenvironment in tumorigenesis. *J Cancer* (2017) 8:761–73. doi:10.7150/jca.17648
95. Mazzone M, Dettori D, de Oliveira RL, Loges S, Schmidt T, Jonckx B, et al. Heterozygous deficiency of PHD2 restores tumor oxygenation and inhibits metastasis via endothelial normalization. *Cell* (2009) 136:839–51. doi:10.1016/j.cell.2009.01.020
96. Kalluri R. The biology and function of fibroblasts in cancer. *Nat Rev Cancer* (2016) 16:582–98. doi:10.1038/nrc.2016.73
97. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* (2005) 121:335–48. doi:10.1016/j.cell.2005.02.034
98. Erdogan B, Webb DJ. Cancer-associated fibroblasts modulate growth factor signaling and extracellular matrix remodeling to regulate tumor metastasis. *Biochem Soc Trans* (2017) 45:229–36. doi:10.1042/BST20160387
99. Kuzet S-E, Gaggioli C. Fibroblast activation in cancer: when seed fertilizes soil. *Cell Tissue Res* (2016) 365:607–19. doi:10.1007/s00441-016-2467-x
100. Kim J, Evans C, Weidemann A, Takeda N, Lee YS, Stockmann C, et al. Loss of fibroblast HIF-1α accelerates tumorigenesis. *Cancer Res* (2012) 72:3187–95. doi:10.1158/0008-5472.CAN-12-0534
101. De Francesco EM, Lappano R, Santolla MF, Marsico S, Caruso A, Maggiolini M. HIF-1α/GPER signaling mediates the expression of VEGF induced by hypoxia in breast cancer associated fibroblasts (CAFs). *Breast Cancer Res* (2013) 15:R64. doi:10.1186/bcr3458
102. Chiavarina B, Whitaker-Menezes D, Migneco G, Martinez-Outschoorn UE, Pavlides S, Howell A, et al. HIF1-alpha functions as a tumor promoter in cancer associated fibroblasts, and as a tumor suppressor in breast cancer cells: autophagy drives compartment-specific oncogenesis. *Cell Cycle* (2010) 9:3534–51. doi:10.4161/cc.9.17.12908
103. Chiavarina B, Martinez-Outschoorn UE, Whitaker-Menezes D, Howell A, Tanowitz HB, Pestell RG, et al. Metabolic reprogramming and two-compartment tumor metabolism: opposing role(s) of HIF1α and HIF2α in tumor-associated fibroblasts and human breast cancer cells. *Cell Cycle* (2012) 11:3280–9. doi:10.4161/cc.21643
104. Madsen CD, Pedersen JT, Venning FA, Singh LB, Moeendarbary E, Charras G, et al. Hypoxia and loss of PHD2 inactivate stromal fibroblasts to decrease tumour stiffness and metastasis. *EMBO Rep* (2015) 16:1394–408. doi:10.15252/embr.201540107
105. Calvo F, Ege N, Grande-Garcia A, Hooper S, Jenkins RP, Chaudhry SI, et al. Mechanotransduction and YAP-dependent matrix remodelling is required for the generation and maintenance of cancer-associated fibroblasts. *Nat Cell Biol* (2013) 15:637–46. doi:10.1038/ncb2756
106. Siemann DW, Horsman MR. Modulation of the tumor vasculature and oxygenation to improve therapy. *Pharmacol Ther* (2015) 153:107–24. doi:10.1016/j.pharmthera.2015.06.006
107. Baker A-M, Bird D, Welti JC, Gourlaouen M, Lang G, Murray GI, et al. Lysyl oxidase plays a critical role in endothelial cell stimulation to drive tumor angiogenesis. *Cancer Res* (2013) 73:583–94. doi:10.1158/0008-5472.CAN-12-2447
108. Carmeliet P, Jain RK. Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. *Nat Rev Drug Discov* (2011) 10:417–27. doi:10.1038/nrd3455
109. Tang N, Wang L, Esko J, Giordano FJ, Huang Y, Gerber H-P, et al. Loss of HIF-1alpha in endothelial cells disrupts a hypoxia-driven VEGF autocrine loop necessary for tumorigenesis. *Cancer Cell* (2004) 6:485–95. doi:10.1016/j.ccr.2004.09.026
110. Branco-Price C, Zhang N, Schnelle M, Evans C, Katschinski DM, Liao D, et al. Endothelial cell HIF-1α and HIF-2α differentially regulate metastatic success. *Cancer Cell* (2012) 21:52–65. doi:10.1016/j.ccr.2011.11.017
111. Skuli N, Liu L, Runge A, Wang T, Yuan L, Patel S, et al. Endothelial deletion of hypoxia-inducible factor-2alpha (HIF-2alpha) alters vascular function and tumor angiogenesis. *Blood* (2009) 114:469–77. doi:10.1182/blood-2008-12-193581
112. Skuli N, Majmundar AJ, Krock BL, Mesquita RC, Mathew LK, Quinn ZL, et al. Endothelial HIF-2α regulates murine pathological angiogenesis and revascularization processes. *J Clin Invest* (2012) 122:1427–43. doi:10.1172/JCI57322
113. Cooke VG, LeBleu VS, Keskin D, Khan Z, O'Connell JT, Teng Y, et al. Pericyte depletion results in hypoxia-associated epithelial-to-mesenchymal transition and metastasis mediated by met signaling pathway. *Cancer Cell* (2012) 21:66–81. doi:10.1016/j.ccr.2011.11.024
114. Keskin D, Kim J, Cooke VG, Wu C-C, Sugimoto H, Gu C, et al. Targeting vascular pericytes in hypoxic tumors increases lung metastasis via angiopoietin-2. *Cell Rep* (2015) 10:1066–81. doi:10.1016/j.celrep.2015.01.035
115. Nielsen SR, Schmid MC. Macrophages as key drivers of cancer progression and metastasis. *Mediators Inflamm* (2017) 2017:9624760. doi:10.1155/2017/9624760
116. Du R, Lu KV, Petritsch C, Liu P, Ganss R, Passegué E, et al. HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. *Cancer Cell* (2008) 13:206–20. doi:10.1016/j.ccr.2008.01.034
117. Casazza A, Laoui D, Wenes M, Rizzolio S, Bassani N, Mambretti M, et al. Impeding macrophage entry into hypoxic tumor areas by Sema3A/Nrp1 signaling blockade inhibits angiogenesis and restores antitumor immunity. *Cancer Cell* (2013) 24:695–709. doi:10.1016/j.ccr.2013.11.007
118. Takeda N, O'Dea EL, Doedens A, Kim J, Weidemann A, Stockmann C, et al. Differential activation and antagonistic function of HIF-{alpha} isoforms in macrophages are essential for NO homeostasis. *Genes Dev* (2010) 24:491–501. doi:10.1101/gad.1881410
119. Werno C, Menrad H, Weigert A, Dehne N, Goerdt S, Schledzewski K, et al. Knockout of HIF-1α in tumor-associated macrophages enhances M2 polarization and attenuates their pro-angiogenic responses. *Carcinogenesis* (2010) 31:1863–72. doi:10.1093/carcin/bgq088
120. Laoui D, Van Overmeire E, Di Conza G, Aldeni C, Keirsse J, Morias Y, et al. Tumor hypoxia does not drive differentiation of tumor-associated macrophages but rather fine-tunes the M2-like macrophage population. *Cancer Res* (2014) 74:24–30. doi:10.1158/0008-5472.CAN-13-1196
121. Doedens AL, Stockmann C, Rubinstein MP, Liao D, Zhang N, DeNardo DG, et al. Macrophage expression of hypoxia-inducible factor-1 alpha suppresses T-cell function and promotes tumor progression. *Cancer Res* (2010) 70:7465–75. doi:10.1158/0008-5472.CAN-10-1439
122. Imtiyaz HZ, Williams EP, Hickey MM, Patel SA, Durham AC, Yuan L-J, et al. Hypoxia-inducible factor 2alpha regulates macrophage function in mouse models of acute and tumor inflammation. *J Clin Invest* (2010) 120:2699–714. doi:10.1172/JCI39506
123. Mamlouk S, Kalucka J, Singh RP, Franke K, Muschter A, Langer A, et al. Loss of prolyl hydroxylase-2 in myeloid cells and T-lymphocytes impairs tumor development. *Int J Cancer* (2014) 134:849–58. doi:10.1002/ijc.28409
124. Roda JM, Wang Y, Sumner LA, Phillips GS, Marsh CB, Eubank TD. Stabilization of HIF-2α induces sVEGFR-1 production from tumor-associated macrophages and decreases tumor growth in a murine melanoma model. *J Immunol* (2012) 189:3168–77. doi:10.4049/jimmunol.1103817
125. Corzo CA, Condamine T, Lu L, Cotter MJ, Youn J-I, Cheng P, et al. HIF-1α regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment. *J Exp Med* (2010) 207:2439–53. doi:10.1084/jem.20100587
126. Sethumadhavan S, Silva M, Philbrook P, Nguyen T, Hatfield SM, Ohta A, et al. Hypoxia and hypoxia-inducible factor (HIF) downregulate antigen-presenting MHC class I molecules limiting tumor cell recognition by T cells. *PLoS One* (2017) 12:e0187314. doi:10.1371/journal.pone.0187314
127. Baker A-M, Cox TR, Bird D, Lang G, Murray GI, Sun X-F, et al. The role of lysyl oxidase in SRC-dependent proliferation and metastasis of colorectal cancer. *J Natl Cancer Inst* (2011) 103:407–24. doi:10.1093/jnci/djq569
128. Lu P, Weaver VM, Werb Z. The extracellular matrix: a dynamic niche in cancer progression. *J Cell Biol* (2012) 196:395–406. doi:10.1083/jcb.201102147
129. Provenzano PP, Inman DR, Eliceiri KW, Knittel JG, Yan L, Rueden CT, et al. Collagen density promotes mammary tumor initiation and progression. *BMC Med* (2008) 6:11. doi:10.1186/1741-7015-6-11
130. Myllyharju J, Schipani E. Extracellular matrix genes as hypoxia-inducible targets. *Cell Tissue Res* (2010) 339:19–29. doi:10.1007/s00441-009-0841-7
131. Barker HE, Bird D, Lang G, Erler JT. Tumor-secreted LOXL2 activates fibroblasts through FAK signaling. *Mol Cancer Res* (2013) 11:1425–36. doi:10.1158/1541-7786.MCR-13-0033-T
132. Gilkes DM, Bajpai S, Chaturvedi P, Wirtz D, Semenza GL. Hypoxia-inducible factor 1 (HIF-1) promotes extracellular matrix remodeling under hypoxic

conditions by inducing P4HA1, P4HA2, and PLOD2 expression in fibroblasts. *J Biol Chem* (2013) 288:10819–29. doi:10.1074/jbc.M112.442939

133. Semenza GL, Jiang BH, Leung SW, Passantino R, Concordet JP, Maire P, et al. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *J Biol Chem* (1996) 271:32529–37. doi:10.1074/jbc.271.51.32529
134. Kim J, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab* (2006) 3:177–85. doi:10.1016/j.cmet.2006.02.002
135. Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. *Cell Metab* (2006) 3:187–97. doi:10.1016/j.cmet.2006.01.012
136. Maxwell PH, Dachs GU, Gleadle JM, Nicholls LG, Harris AL, Stratford IJ, et al. Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. *Proc Natl Acad Sci U S A* (1997) 94:8104–9. doi:10.1073/pnas.94.15.8104
137. Chen C, Pore N, Behrooz A, Ismail-Beigi F, Maity A. Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. *J Biol Chem* (2001) 276:9519–25. doi:10.1074/jbc.M010144200
138. Lou Y, McDonald PC, Oloumi A, Chia S, Ostlund C, Ahmadi A, et al. Targeting tumor hypoxia: suppression of breast tumor growth and metastasis by novel carbonic anhydrase IX inhibitors. *Cancer Res* (2011) 71:3364–76. doi:10.1158/0008-5472.CAN-10-4261
139. Chan SY, Zhang Y-Y, Hemann C, Mahoney CE, Zweier JL, Loscalzo J. MicroRNA-210 controls mitochondrial metabolism during hypoxia by repressing the iron-sulfur cluster assembly proteins ISCU1/2. *Cell Metab* (2009) 10:273–84. doi:10.1016/j.cmet.2009.08.015
140. Chen Z, Li Y, Zhang H, Huang P, Luthra R. Hypoxia-regulated microRNA-210 modulates mitochondrial function and decreases ISCU and COX10 expression. *Oncogene* (2010) 29:4362–8. doi:10.1038/onc.2010.193
141. Puisségur MP, Mazure NM, Bertero T, Pradelli L, Grosso S, Robbe-Sermesant K, et al. miR-210 is overexpressed in late stages of lung cancer and mediates mitochondrial alterations associated with modulation of HIF-1 activity. *Cell Death Differ* (2011) 18:465–78. doi:10.1038/cdd.2010.119
142. Kikuchi D, Minamishima YA, Nakayama K. Prolyl-hydroxylase PHD3 interacts with pyruvate dehydrogenase (PDH)-E1β and regulates the cellular PDH activity. *Biochem Biophys Res Commun* (2014) 451:288–94. doi:10.1016/j.bbrc.2014.07.114
143. Zhang H, Bosch-Marce M, Shimoda LA, Tan YS, Baek JH, Wesley JB, et al. Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. *J Biol Chem* (2008) 283:10892–903. doi:10.1074/jbc.M800102200
144. Movafagh S, Crook S, Vo K. Regulation of hypoxia-inducible factor-1a by reactive oxygen species: new developments in an old debate. *J Cell Biochem* (2015) 116:696–703. doi:10.1002/jcb.25074
145. Sasabe E, Yang Z, Ohno S, Yamamoto T. Reactive oxygen species produced by the knockdown of manganese-superoxide dismutase up-regulate hypoxia-inducible factor-1alpha expression in oral squamous cell carcinoma cells. *Free Radic Biol Med* (2010) 48:1321–9. doi:10.1016/j.freeradbiomed.2010.02.013
146. Du J, Xu R, Hu Z, Tian Y, Zhu Y, Gu L, et al. PI3K and ERK-induced Rac1 activation mediates hypoxia-induced HIF-1α expression in MCF-7 breast cancer cells. *PLoS One* (2011) 6:e25213. doi:10.1371/journal.pone.0025213
147. Semenza GL. Hypoxia-inducible factors: coupling glucose metabolism and redox regulation with induction of the breast cancer stem cell phenotype. *EMBO J* (2017) 36:252–9. doi:10.15252/embj.201695204
148. Lyssiotis CA, Kimmelman AC. Metabolic interactions in the tumor microenvironment. *Trends Cell Biol* (2017) 27:863–75. doi:10.1016/j.tcb.2017.06.003
149. Zhang D, Wang Y, Shi Z, Liu J, Sun P, Hou X, et al. Metabolic reprogramming of cancer-associated fibroblasts by IDH3α downregulation. *Cell Rep* (2015) 10:1335–48. doi:10.1016/j.celrep.2015.02.006

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 07 June 2018
doi: 10.3389/fonc.2018.00210

Activation of p62/SQSTM1–Keap1–Nuclear Factor Erythroid 2-Related Factor 2 Pathway in Cancer

Yoshinobu Ichimura and Masaaki Komatsu*

Department of Biochemistry, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

Autophagy and the Keap1–Nrf2 system are major cellular defense mechanisms against metabolic and oxidative stress. These two systems are linked *via* phosphorylation of the ubiquitin binding autophagy receptor protein p62/SQSTM1 in the p62–Keap1–Nrf2 pathway. The p62–Keap1–Nrf2 pathway plays a protective role in normal cells; however, recent studies indicate that this pathway induces tumorigenesis of pre-malignant cells, and promotes the growth and drug resistance of tumor cells *via* metabolic reprogramming mediated by Nrf2 activation. These findings suggest that impairment of autophagy is involved in the acquisition of malignancy and maintenance of tumors, and furthermore, that p62/SQSTM1 could be a potential target for chemotherapy in cancers that harbor excess p62.

Keywords: selective autophagy, p62/SQSTM1, Keap1–nuclear factor erythroid 2-related factor 2 system, metabolic reprogramming, cancer

OPEN ACCESS

Edited by:
Giuseppe Filomeni,
Danish Cancer Society, Denmark

Reviewed by:
Manuela D'Eletto,
Università degli Studi di Roma Tor Vergata, Italy
Hilary Ann Coller,
University of California, Los Angeles, United States
Gianmaria Fimia,
University of Salento, Italy

****Correspondence:***
Yoshinobu Ichimura
yichimu@med.niigata-u.ac.jp

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *22 March 2018*
Accepted: *24 May 2018*
Published: *07 June 2018*

Citation:
Ichimura Y and Komatsu M (2018) Activation of p62/SQSTM1–Keap1–Nuclear Factor Erythroid 2-Related Factor 2 Pathway in Cancer. Front. Oncol. 8:210. doi: 10.3389/fonc.2018.00210

INTRODUCTION

Autophagy is a bulk degradation process in which cytoplasmic components are sequestered in a double-membrane structure to form an autophagosome. The contents of the autophagosome are subsequently degraded after fusion with a lysosome (**Figure 1**) (1). Under starvation conditions, autophagy provides amino acids essential for protein synthesis in response to metabolic stress. Basal autophagy, on the other hand, removes specific substrates, including protein aggregates, damaged organelles, and invading bacteria. This selective pathway uses autophagic receptor proteins for efficient degradation. Autophagic receptors are typically categorized into two different types: ubiquitin binding proteins and organelle membrane proteins. Ubiquitin binding receptors include p62/SQSTM1, neighbor of BRCA1 gene (NBR1), optineurin, nuclear dot protein 52 kDa/calcium binding and coiled-coil domain2 (CALCOCO2), Tax1 binding protein 1, and toll-interacting protein; membrane binding receptors include BCL2/adenovirus E1B 19 kDa interacting protein 3-like (NIX/BNIP3L), BNIP3, FUN14 domain containing 1, and family with sequence similarity 134, member B (FAM134B) (2). These receptors recognize and sort substrates, and recruit core autophagic machinery to the target at existing autophagosome formation sites.

Conserved among metazoans, the ubiquitin binding autophagy receptor p62/SQSTM1 (hereafter referred to as p62) acts as a hub protein in various cellular signaling pathways, including NF-κB, mechanistic target of rapamycin (mTOR), Caspase 8, and nuclear factor erythroid 2-related factor 2 (Nrf2) (3–7). p62 mediates the autophagic degradation of polyubiquitinated substrates *via* direct interaction with microtubule-associated protein light chain 3 (LC3) on the autophagosome (2) (**Figure 1**). p62 forms aggregates by self-oligomerization of its N-terminal Phox and Bem1 (PB1) domain, and associates with ubiquitin through its C-terminal ubiquitin-associated (UBA) domain (**Figure 2**). NBR1 has a remarkable similarity with p62 in the domain architecture, which consists of PB1, zinc finger, LC3-interacting region (LIR), and UBA domains (8). PB1 domain of p62 forms

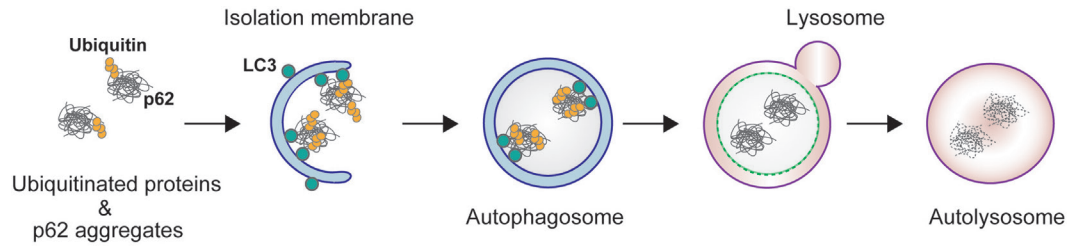


FIGURE 1 | p62-mediated selective autophagy. p62 interacts with ubiquitinated proteins through its C-terminal ubiquitin-associated (UBA) domain, and forms aggregates through its N-terminal Phox and Bem1 domain. The resulting protein aggregates are tethered to the autophagosome by direct interaction of p62 and light chain 3 localized on the isolation membrane. The isolation membrane elongates and sequesters p62 and ubiquitin into the autophagosome. The autophagosome fuses with lysosome to form an autolysosome.

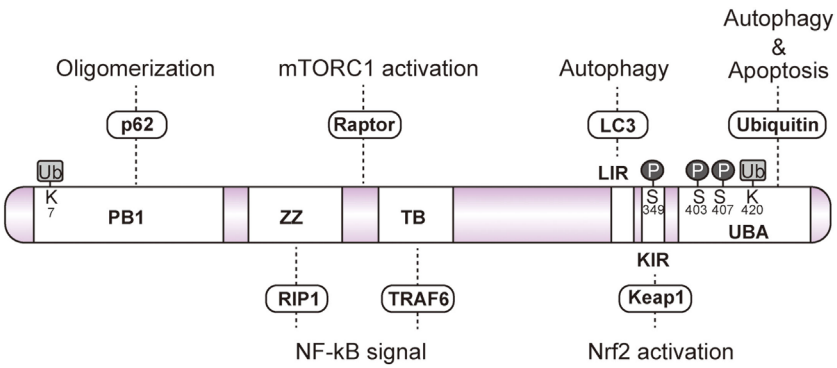


FIGURE 2 | Schematic domain of p62. p62 forms a self-oligomer through its Phox and Bem1 (PB1) domain. The zinc finger (ZZ) and TRAF6-binding domains (TB) interact with RIP1 and TRAF6, respectively, to regulate NF-κB signaling. Raptor interacts with the unidentified region between ZZ and TB to activate mechanistic target of rapamycin complex 1. Light chain 3-interacting region and the C-terminal ubiquitin-associated domain (UBA) are essential for the sequestration of ubiquitinated substrates into the autophagosome. Phosphorylation of serine 403 and serine 407 residues in the UBA domain facilitates the interaction with ubiquitin. Phosphorylation of Keap1-interacting region at serine 349 enhances the interaction with Keap1. The UBA domain interacts with ubiquitinated caspase8 to induce apoptosis. Ubiquitination of the PB1 domain at lysine 7 abrogates p62 oligomerization. Ubiquitination of the UBA domain at lysine 420 disrupts the interaction with the ubiquitin chain.

self-oligomer or hetero-oligomer with other PB1 proteins including NBR1, while PB1 domain of NBR1 forms hetero-oligomer only. Thus, NBR1 self-interacts through their coiled-coil domain, and cooperates with p62 oligomer in selective autophagy of ubiquitinated substrates (9). There are several examples of post-translational modifications of p62 for selective autophagy. For example, phosphorylation of the p62 UBA domain at serine 407 by unc-51-like kinase 1 (ULK1) destabilizes the UBA dimer of p62, while sequential phosphorylation of serine 403 by casein kinase 2 (CK2), TANK-binding kinase 1 (TBK1), or ULK1 increases the affinity of UBA for ubiquitin chains (10–12). Ubiquitination of p62 at lysine 420 by the Keap1/Cul3 E3 ligase complex inhibits the dimerization of the UBA domain (13), while ubiquitination at lysine 7 in the PB1 domain by the E3 ligase TRIM21 suppresses the oligomerization of p62 (14).

Upon various cellular stress conditions, p62 functions as a signaling hub *via* characteristic domains, such as the zinc finger (ZZ) domain, TRAF6 binding (TB) domain, LIR, and Keap1-interacting region (KIR), which interacts with RIP kinase, TRAF6, Raptor, LC3, and Keap1, respectively (**Figure 2**). Transcription of p62 is activated by cellular stresses, such as oxidative, metabolic, and pathogenic conditions, and aberrant expression of p62 or defective autophagy causes appearance of large number of p62 aggregates into the cytoplasm (15, 16). Importantly, the aggregate of p62 has been found in a common hallmark in some of serious diseases, such as cancer, alcoholic hepatitis, and neurodegenerative disease (17). The aforementioned diseases are thought to result from loss or dysfunction of p62-regulated cell signaling.

PATHOPHYSIOLOGICAL ROLE OF AUTOPHAGY IN CANCER

Early research has indicated a role of autophagy in tumor suppression. Beclin 1, the mammalian ortholog of yeast Atg6, was reported as autophagy-related protein involved in tumorigenesis (18). Specifically, beclin 1 heterozygous-deficient mice were shown to exhibit increased frequency of spontaneous tumorigenesis in the liver, lung, and lymphomas (19, 20). Because systemic knockout of *Atg* genes in mice exhibit neonatal lethality due to deprivation of amino acids in plasma and tissue (21), conditional knockouts of *Atg* genes in mice have been used as a physiological

model of autophagy. Intriguingly, mosaic knockouts of *Atg5* or liver-specific knockouts of *Atg7* in mice have demonstrated that autophagy deficiency generates benign tumors in the liver in an age-dependent manner (22, 23). Impairment of autophagy results in accumulation of damaged mitochondria, which are major sources of reactive oxygen species (ROS) resulting in DNA damage, thus contributing to malignant progression (24). Other studies report that reduction of autophagy results in growth suppression of various cancers, such as hepatocyte, pancreatic cancer, lung cancer, breast cancer, colon cancer driven by K-Ras or BRAF mutations (25). On the other hand, studies have found that overexpression of transcription factor EB, a critical regulator of autophagy, promotes cancer growth (26, 27). Autophagy-deficient tumors re-growth and form large tumors in allograft, suggesting that growth arrest of autophagy-deficient tumors is canceled through nutrient-generating autophagy in an ectopic environment (28). It has been reported recently that oxidation of p62 promotes its oligomerization *via* disulfide-linked conjugates, followed by activation of autophagy (29). In this pathway, p62 senses the ROS and induces autophagy for cellular homeostasis and cell survival even under the oxidative stress conditions in aging or cancer. Moreover, increased p62 in autophagy-defective cells inhibits RNF168, an E3 ligase for histone H2A activated in response to DNA damage (30). These findings suggest that autophagy-deficient cells abolish DNA repair activity thereby resulting in tumorigenesis. Autophagy plays a complex role in cancer, and its function can be dependent on the stage, environment, or type of cancer. Taken together, the above findings indicate that while autophagy plays a role in the inhibition of tumorigenesis, it could also facilitate the tumor growth once established, at least in mouse models.

Keap1–Nrf2 PATHWAY

Keap1–Nrf2 pathway is a critical cytoprotective response mediated by the activation of transcription factor Nrf2 during oxidative or electrophilic stress. Under normal conditions, Nrf2 is constitutively degraded in ubiquitin-proteasome system *via* the interaction with the E3 ubiquitin ligase adaptor protein Kelch-like erythroid cell-derived protein with CNC homology [ECH]-associated protein 1 (Keap1) (31). Keap1 binds to Nrf2 through direct interaction between the double glycine repeat or Kelch repeat (DGR) and the C-terminal region (CTR) of Keap1 (Keap1DC), and the ETGE and DLGex motifs of Nrf2. Known as the hinge and latch model (32–34), Keap1 forms a homodimer with its N-terminal BTB domain in which one protein interacts with high affinity to ETGE and the other with low affinity to DLGex (**Figure 3A**). Oxidative stress or electrophiles trigger a conformational change of Keap1 by modification of certain cysteine residues in Keap1,

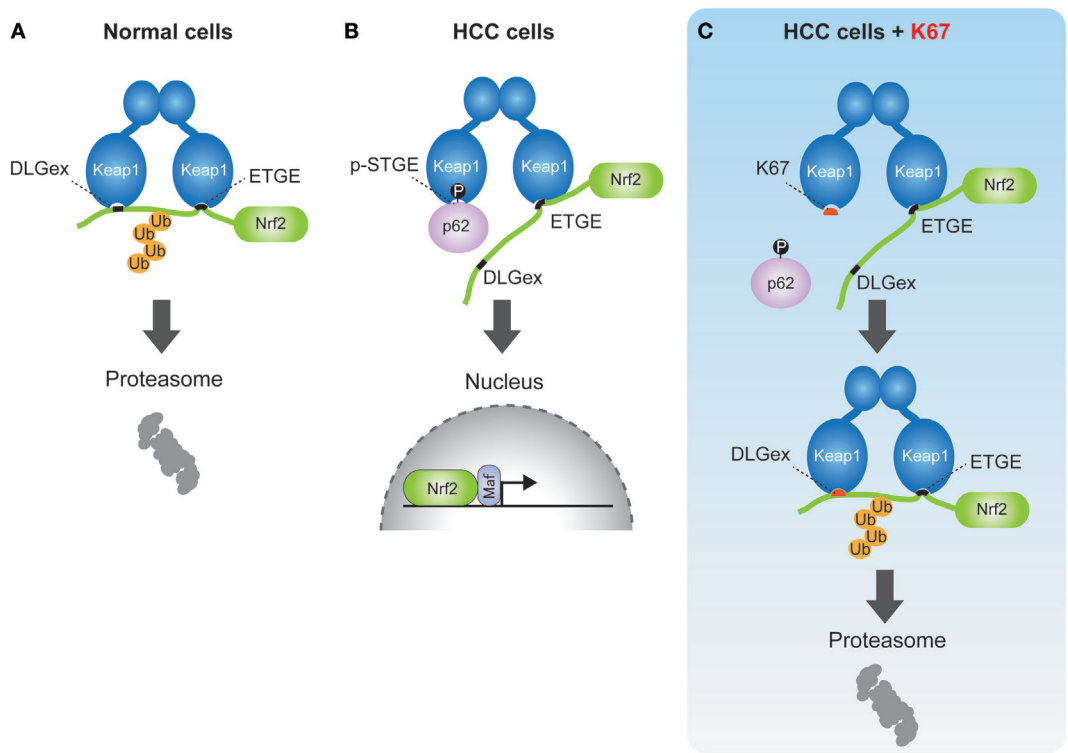


FIGURE 3 | Schematic model of nuclear factor erythroid 2-related factor 2 (Nrf2) activation by phospho-p62 **(A)**. In normal cells, the ETGE and DLGex motifs of Nrf2 bind to the Keap1DC domain, which promotes ubiquitination of Nrf2 followed by its proteasomal degradation. **(B)** In human hepatocellular carcinoma cells, phospho-p62 (pS349) competitively interacts with the DLGex site on Keap1DC, resulting in the translocation of Nrf2 into nucleus. **(C)** K67 binds to Keap1DC at higher affinity than phosphor-p62, whereas the DLGex motif of Nrf2 binds to Keap1DC with covering K67. Nrf2 is eventually ubiquitinated and degraded by the proteasome.

which leads to its dissociation from Nrf2 (35). The released Nrf2 translocates into the nucleus to induce the transcription of antioxidant-responsive element-regulated genes, such as the cytoprotective or metabolic-related genes *NQO1*, *HO-1*, *GCLC*, *GSTm*, which help to protect the cells from oxidative and metabolic stress.

p62–Keap1–Nrf2 PATHWAY

In previous reports, we demonstrated that phosphorylation of p62 at serine 349 results in Nrf2 activation (6). p62 also has a KIR motif (349-STGE-352) (36), which allows binding to Keap1DC, but its affinity to Keap1 is significantly lower compared to the ETGE motif of Nrf2. During selective autophagy, p62 is translocated to ubiquitinated targets, such as protein aggregates, depolarized mitochondria, and pathogens, through the phosphorylation of the p62 UBA domain at serine 403 and serine 407 by CK2 and/or TBK1 (10, 11). Subsequently, mTOR complex 1 (mTORC1) phosphorylates p62 at serine 349, which dramatically enhances its interaction with Keap1, since the Keap1 binding affinity of phospho-p62 is higher than that of DLGex motif of Nrf2 (**Figure 3B**). Finally, the DLGex of Nrf2 is competitively displaced by phospho-p62, which results in the dissociation of Nrf2 from Keap1 and robust activation of Nrf2 (p62–Keap1–Nrf2 pathway) (**Figure 3B**) (6). p62–Keap1 and ubiquitinated cargos are eventually removed by selective autophagy. These results indicate that p62-mediated selective autophagy is coupled with the Keap1-Nrf2 system in normal cells. In p62–Keap1–Nrf2 pathway, the cytoprotective effects of Nrf2 activation could be enhanced in concert with the selective degradation of phosphorylated p62 and Keap1 complex.

DYSREGULATION OF THE p62–Keap1–Nrf2 PATHWAY IN CANCER

Notably, persistent phosphorylation of mouse p62 at serine 351 (corresponding to serine 349 in human p62) has been found in hepatic adenoma in autophagy-deficient livers and in hepatitis C virus-positive human hepatocellular carcinoma (HCC) (22, 37). Knockout of *p62* in an HCC cell line markedly abrogates tumor growth, whereas forced expression of a phosphorylation-mimic

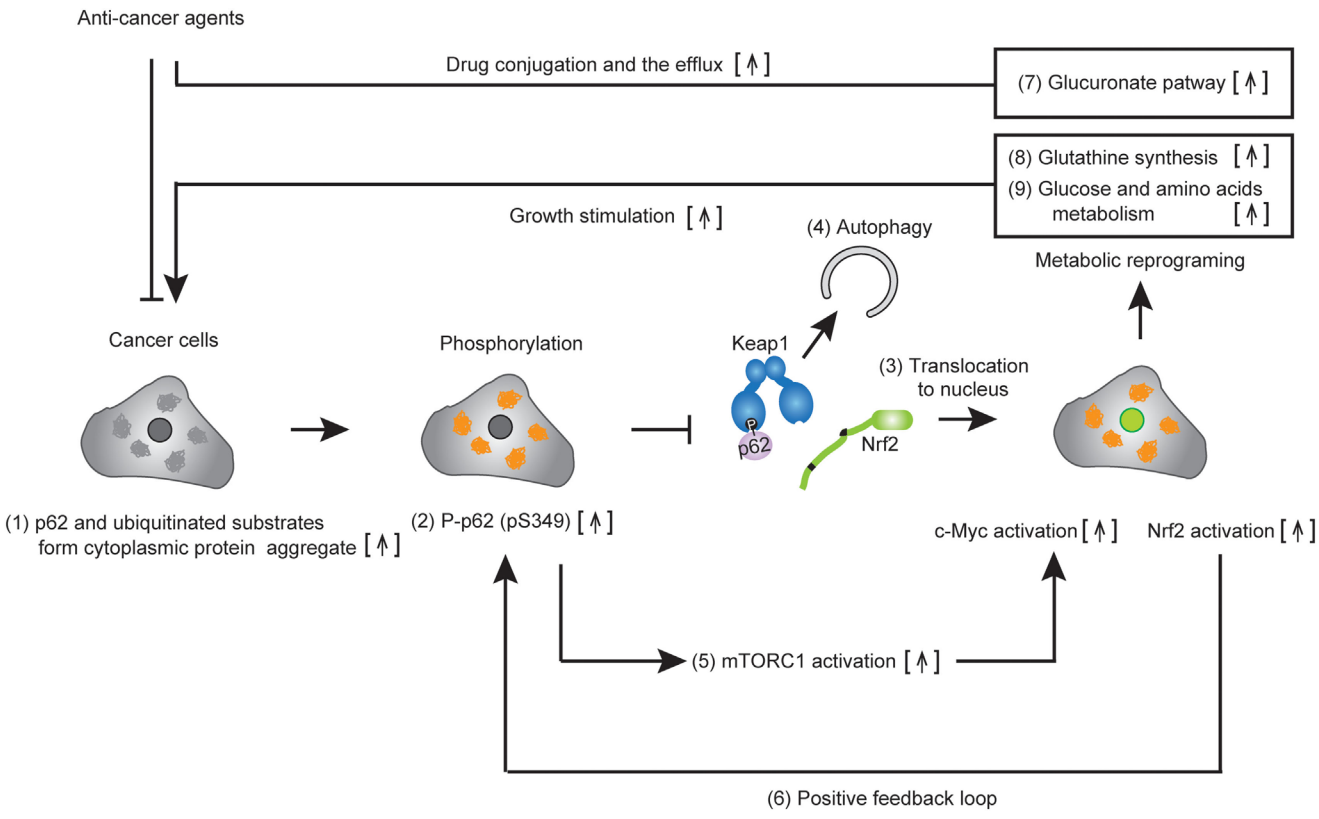


FIGURE 4 | Metabolic reprogramming of cancer cells harboring phospho-p62. Accumulated p62 and ubiquitinated substrates form cytoplasmic protein aggregates in established tumor cells (1). The p62 localized in the protein aggregates are phosphorylated at serine 349 (2). Then, DLGex of nuclear factor erythroid 2-related factor 2 (Nrf2) is competitively displaced by phosphop-p62, which causes the dissociation of Nrf2 from Keap1. Nrf2 translocates to the nucleus and activates Nrf2-mediated gene expression (3), while the resulting p62–Keap1 complex is removed by p62-dependent selective autophagy (4). Increased p62 positively regulates c-Myc activity *via* the activation of mechanistic target of rapamycin complex 1 (5). Persistent activation of Nrf2 occurs due to the positive feedback loop of Nrf2 in cancer cells (6). Nrf2 activation induces metabolic reprogramming, including the glucuronate pathway (7) and glutathione synthesis (8), while c-Myc enhances glucose and glutamine metabolism (9). The activation of multiple metabolic pathways permits the drug resistance and cell proliferation of cancer.

allele of p62, but not a phosphorylation defective mutant, resulted in recovery of the growth defect (6, 22). These results indicate that the persistent activation of Nrf2 through phosphorylation of p62 is involved in the development of HCC. Importantly, Nrf2 also induces *p62* expression, resulting in the persistent activation of Nrf2 *via* a positive feedback loop in the p62–Nrf2–Keap1 pathway (16). Consistently, an amplified copy number of *p62* on chromosome 5q has been identified in renal cancer, suggesting that *p62* is an oncogene. Furthermore, accumulating evidence demonstrates that the abnormal expression of p62 is associated with malignancy in various cancers, including liver (22, 37), kidney (38, 39), lung (40), breast (41, 42), pancreatic (43), prostate (44, 45), head and neck (46, 47), ovarian (48, 49), oral (50, 51), colon (52, 53), endometrial (54), skin (55, 56), and gastric cancers (57). Indeed, accumulation of phosphorylated p62 has been observed in about half of HCC patients in our studies (6). Somatic mutations of Nrf2 and Keap1 have also been found in cancers at high frequency (58–60); these mutations could cause persistent activation of Nrf2 *via* disrupting the interaction between Nrf2 and Keap1. The above-mentioned lines of evidence suggest that dysregulation of p62–Keap1–Nrf2 pathway is involved in cancer development.

METABOLIC REPROGRAMMING BY THE p62–Keap1–Nrf2 PATHWAY

Nuclear factor erythroid 2-related factor 2 has been shown to regulate the expression of antioxidant proteins, detoxification enzymes, proteasome subunits, and autophagy-related proteins for oxidative stress response and proteostasis (protein homeostasis) in normal cells. Recent studies demonstrate that Nrf2 increases the expression of multiple enzymes involved in the pentose phosphate pathway, purine nucleotide synthesis, as well as glutathione synthesis, and glutaminolysis in lung cancer, which activates the phosphatidylinositol 3-kinase-Akt pathway (61). In support of these findings, we also found that Nrf2 activation provided metabolic reprogramming of glucose and glutamine through the activation of Nrf2 in HCC harboring phosphorylated p62 (p-S349) (**Figure 3B**), which led to increased cell proliferation and resistance to anti-cancer agents of HCC (37) (**Figure 4**). Taken together, these findings suggest that molecular targeting of p62 represents a potential chemotherapeutic approach against HCC.

By chemical screening, we have identified an inhibitor for the Keap1-phosphorylated p62 (p-S349) protein–protein interaction—the acetonyl naphthalene derivative K67 (37) (**Figure 3C**). Structural analysis demonstrated that K67 binds to a Keap1DC pocket, which is the binding site of phosphorylated p62, Nrf2-ETGE, and Nrf2-DLGex. Treatment of HCC with K67 suppressed proliferation and reduced tolerance to cisplatin or sorafenib (37). Levels of p62 accumulation and c-Myc expression are reportedly associated with high risk for tumor recurrence and poor prognosis of HCC patients (62). Further, it has been demonstrated that high p62 expression in non-tumor tissue is required for transformation to HCC, which was caused by the activation of Nrf2, mTORC1, and c-Myc (62) (**Figure 4**). These results are consistent with the anti-proliferative and anti-malignant effects found in autophagy-deficient tumor cells (63). More recently, Karin and colleagues reported that p62-mediated activation of Nrf2 triggers mouse double minute 2 homolog (MDM2) expression in premalignant pancreatic intraepithelial neoplasia 1, resulting in development and malignancy of pancreatic ductal adenocarcinoma (43) (**Figure 5**).

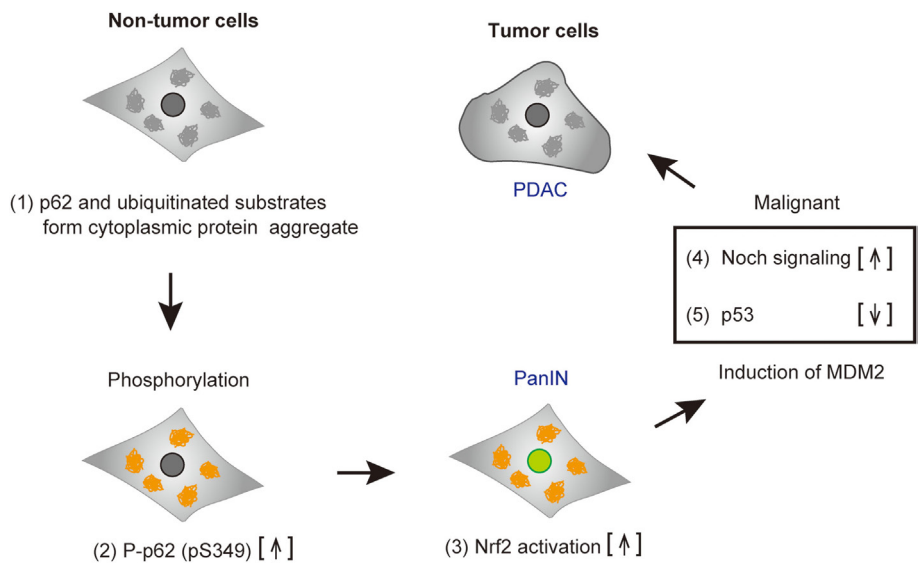


FIGURE 5 | Tumorigenesis of cancer cells harboring phospho-p62. Accumulated p62 and ubiquitinated substrates form cytoplasmic protein aggregates in pre-malignant tumor cells (1). p62 is phosphorylated (2), and subsequently nuclear factor erythroid 2-related factor 2 (Nrf2) is activated (3). Mouse double minute 2 homolog (MDM2) induced by Nrf2 results in the increase of Notch signaling (4) and the reduction of p53, followed by cell malignancy (5). Abbreviations: PanIN, premalignant pancreatic intraepithelial neoplasia; PDAC, pancreatic ductal adenocarcinoma.

CONCLUSION AND FUTURE PERSPECTIVES

In recent years, there are many reports showing that p62–Keap1–Nrf2 pathway plays a protective role in oxidative and stress conditions. For example, ER stress-induced apoptosis is prevented by the activation of p62–Keap1–Nrf2 pathway (64). Quercetin attenuates the hepatotoxicity induced by a various hepatotoxicants, through the activation of p62–Keap1–Nrf2 pathway (65). Licochalcone A activates p62–Keap1–Nrf2 pathway and suppresses arthritis in a collagen-induced arthritis mouse model (66). Trehalose induces p62 expression and activates p62–Keap1–Nrf2-mediated antioxidant response during oxidative stress (67). Meanwhile, persistent activation of p62–Keap1–Nrf2 pathway has been shown to be involved in liver tumorigenesis in mice (6, 22). More recently, we demonstrated that overexpression of a p62 variant lacking KIR mitigates Nrf2 activation (68). These results suggest that phopsho-p62 could be a novel target for cancer therapy as described in Ref. (37). Many kinase inhibitors have been used in cancer therapy. However, the protein kinases responsible for the phosphorylation of p62 in tumor have not yet been identified. Further studies are required to identify the regulatory factors involved in the p62–Keap1–Nrf2 pathway.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

YI is supported by a Grant-in-Aid for Scientific Research (B) (16H05137, to YI). MK is supported by a Grant-in-Aid for Scientific Research on Innovative Areas (25111006, 25111001, and 15K21749 to MK), a Japan Society for the Promotion of Science (an A3 foresight program, to MK), and Japan Agency for Medical Research and Development (AMED) (17cm0106101h0002 to MK).

REFERENCES

1. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. *Cell* (2011) 147(4):728–41. doi:10.1016/j.cell.2011.10.026
2. Rogov V, Dotsch V, Johansen T, Kirkin V. Interactions between autophagy receptors and ubiquitin-like proteins form the molecular basis for selective autophagy. *Mol Cell* (2014) 53(2):167–78. doi:10.1016/j.molcel.2013.12.014
3. Sanz L, Diaz-Meco MT, Nakano H, Moscat J. The atypical PKC-interacting protein p62 channels NF-kappaB activation by the IL-1-TRAF6 pathway. *EMBO J* (2000) 19(7):1576–86. doi:10.1093/emboj/19.7.1576
4. Duran A, Serrano M, Leitges M, Flores JM, Picard S, Brown JP, et al. The atypical PKC-interacting protein p62 is an important mediator of RANK-activated osteoclastogenesis. *Dev Cell* (2004) 6(2):303–9. doi:10.1016/S1534-5807(03)00403-9
5. Jin Z, Li Y, Pitti R, Lawrence D, Pham VC, Lill JR, et al. Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. *Cell* (2009) 137(4):721–35. doi:10.1016/j.cell.2009.03.015
6. Ichimura Y, Waguri S, Sou YS, Kageyama S, Hasegawa J, Ishimura R, et al. Phosphorylation of p62 activates the Keap1-Nrf2 pathway during selective autophagy. *Mol Cell* (2013) 51(5):618–31. doi:10.1016/j.molcel.2013.08.003
7. Linares JF, Duran A, Yajima T, Pasparakis M, Moscat J, Diaz-Meco MT. K63 polyubiquitination and activation of mTOR by the p62-TRAF6 complex in nutrient-activated cells. *Mol Cell* (2013) 51(3):283–96. doi:10.1016/j.molcel.2013.06.020
8. Kirkin V, McEwan DG, Novak I, Dikic I. A role for ubiquitin in selective autophagy. *Mol Cell* (2009) 34(3):259–69. doi:10.1016/j.molcel.2009.04.026
9. Waters S, Marchbank K, Solomon E, Whitehouse C, Gautel M. Interactions with LC3 and polyubiquitin chains link nbr1 to autophagic protein turnover. *FEBS Lett* (2009) 583(12):1846–52. doi:10.1016/j.febslet.2009.04.049
10. Matsumoto G, Wada K, Okuno M, Kurosawa M, Nukina N. Serine 403 phosphorylation of p62/SQSTM1 regulates selective autophagic clearance of ubiquitinated proteins. *Mol Cell* (2011) 44(2):279–89. doi:10.1016/j.molcel.2011.07.039
11. Pilli M, Arko-Mensah J, Ponpuak M, Roberts E, Master S, Mandell MA, et al. TBK-1 promotes autophagy-mediated antimicrobial defense by controlling autophagosome maturation. *Immunity* (2012) 37(2):223–34. doi:10.1016/j.immuni.2012.04.015
12. Lim J, Lachenmayer ML, Wu S, Liu W, Kundu M, Wang R, et al. Proteotoxic stress induces phosphorylation of p62/SQSTM1 by ULK1 to regulate selective autophagic clearance of protein aggregates. *PLoS Genet* (2015) 11(2):e1004987. doi:10.1371/journal.pgen.1004987
13. Lee Y, Chou TF, Pittman SK, Keith AL, Razani B, Weihl CC. Keap1/Cullin3 modulates p62/SQSTM1 activity via UBA domain ubiquitination. *Cell Rep* (2017) 19(1):188–202. doi:10.1016/j.celrep.2017.03.030
14. Pan JA, Sun Y, Jiang YP, Bott AJ, Jaber N, Dou Z, et al. TRIM21 ubiquitylates SQSTM1/p62 and suppresses protein sequestration to regulate redox homeostasis. *Mol Cell* (2016) 61(5):720–33. doi:10.1016/j.molcel.2016.02.007
15. Komatsu M, Waguri S, Koike M, Sou YS, Ueno T, Hara T, et al. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. *Cell* (2007) 131(6):1149–63. doi:10.1016/j.cell.2007.10.035
16. Jain A, Lamark T, Sjottem E, Larsen KB, Awuh JA, Overvatn A, et al. p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. *J Biol Chem* (2010) 285(29):22576–91. doi:10.1074/jbc.M110.118976
17. Zatloukal K, Stumptner C, Fuchsbichler A, Heid H, Schnoelzer M, Kenner L, et al. p62 Is a common component of cytoplasmic inclusions in protein aggregation diseases. *Am J Pathol* (2002) 160(1):255–63. doi:10.1016/S0002-9440(10)64369-6
18. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* (1999) 402(6762):672–6. doi:10.1038/45257
19. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, et al. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest* (2003) 112(12):1809–20. doi:10.1172/JCI20039
20. Yue Z, Jin S, Yang C, Levine AJ, Heintz N. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc Natl Acad Sci U S A* (2003) 100(25):15077–82. doi:10.1073/pnas.2436255100
21. Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, et al. The role of autophagy during the early neonatal starvation period. *Nature* (2004) 432(7020):1032–6. doi:10.1038/nature03029
22. Inami Y, Waguri S, Sakamoto A, Kouno T, Nakada K, Hino O, et al. Persistent activation of Nrf2 through p62 in hepatocellular carcinoma cells. *J Cell Biol* (2011) 193(2):275–84. doi:10.1083/jcb.201102031
23. Takamura A, Komatsu M, Hara T, Sakamoto A, Kishi C, Waguri S, et al. Autophagy-deficient mice develop multiple liver tumors. *Genes Dev* (2011) 25(8):795–800. doi:10.1101/gad.2016211
24. Kongara S, Karantza V. The interplay between autophagy and ROS in tumorigenesis. *Front Oncol* (2012) 2:171. doi:10.3389/fonc.2012.00171
25. Towers CG, Thorburn A. Therapeutic targeting of autophagy. *EBioMedicine* (2016) 14:15–23. doi:10.1016/j.ebiom.2016.10.034
26. Settembre C, Di Malta C, Polito VA, Garcia Arencibia M, Vetrini F, Erdin S, et al. TFEB links autophagy to lysosomal biogenesis. *Science* (2011) 332(6036):1429–33. doi:10.1126/science.1204592

27. Wei H, Wang C, Croce CM, Guan JL. p62/SQSTM1 synergizes with autophagy for tumor growth in vivo. *Genes Dev* (2014) 28(11):1204–16. doi:10.1101/gad.237354.113
28. Katheder NS, Khezri R, O'Farrell F, Schultz SW, Jain A, Rahman MM, et al. Microenvironmental autophagy promotes tumour growth. *Nature* (2017) 541(7637):417–20. doi:10.1038/nature20815
29. Carroll B, Otten EG, Manni D, Stefanatos R, Menzies FM, Smith GR, et al. Oxidation of SQSTM1/p62 mediates the link between redox state and protein homeostasis. *Nat Commun* (2018) 9(1):256. doi:10.1038/s41467-017-02746-z
30. Wang Y, Zhang N, Zhang L, Li R, Fu W, Ma K, et al. Autophagy Regulates chromatin ubiquitination in DNA damage response through elimination of SQSTM1/p62. *Mol Cell* (2016) 63(1):34–48. doi:10.1016/j.molcel.2016.05.027
31. Suzuki T, Yamamoto M. Stress-sensing mechanisms and the physiological roles of the Keap1-Nrf2 system during cellular stress. *J Biol Chem* (2017) 292(41):16817–24. doi:10.1074/jbc.R117.800169
32. McMahon M, Thomas N, Itoh K, Yamamoto M, Hayes JD. Dimerization of substrate adaptors can facilitate cullin-mediated ubiquitylation of proteins by a "tethering" mechanism: a two-site interaction model for the Nrf2-Keap1 complex. *J Biol Chem* (2006) 281(34):24756–68. doi:10.1074/jbc.M601119200
33. Tong KI, Katoh Y, Kusunoki H, Itoh K, Tanaka T, Yamamoto M. Keap1 recruits Neh2 through binding to ETGE and DLG motifs: characterization of the two-site molecular recognition model. *Mol Cell Biol* (2006) 26(8):2887–900. doi:10.1128/MCB.26.8.2887-2900.2006
34. Tong KI, Kobayashi A, Katsuoka F, Yamamoto M. Two-site substrate recognition model for the Keap1-Nrf2 system: a hinge and latch mechanism. *Biol Chem* (2006) 387(10–11):1311–20. doi:10.1515/BC.2006.164
35. Taguchi K, Motohashi H, Yamamoto M. Molecular mechanisms of the Keap1-Nrf2 pathway in stress response and cancer evolution. *Genes Cells* (2011) 16(2):123–40. doi:10.1111/j.1365-2443.2010.01473.x
36. Komatsu M, Kurokawa H, Waguri S, Taguchi K, Kobayashi A, Ichimura Y, et al. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nat Cell Biol* (2010) 12(3):213–23. doi:10.1038/ncb2021
37. Saito T, Ichimura Y, Taguchi K, Suzuki T, Mizushima T, Takagi K, et al. p62/Sqstm1 promotes malignancy of HCV-positive hepatocellular carcinoma through Nrf2-dependent metabolic reprogramming. *Nat Commun* (2016) 7:12030. doi:10.1038/ncomms12030
38. Li L, Shen C, Nakamura E, Ando K, Signoretti S, Beroukhim R, et al. SQSTM1 is a pathogenic target of 5q copy number gains in kidney cancer. *Cancer Cell* (2013) 24(6):738–50. doi:10.1016/j.ccr.2013.10.025
39. Lam HC, Baglini CV, Lope AL, Parkhitko AA, Liu HJ, Alesi N, et al. p62/SQSTM1 cooperates with hyperactive mTORC1 to regulate glutathione production, maintain mitochondrial integrity, and promote tumorigenesis. *Cancer Res* (2017) 77(12):3255–67. doi:10.1158/0008-5472.CAN-16-2458
40. Inoue D, Suzuki T, Mitsuishi Y, Miki Y, Suzuki S, Sugawara S, et al. Accumulation of p62/SQSTM1 is associated with poor prognosis in patients with lung adenocarcinoma. *Cancer Sci* (2012) 103(4):760–6. doi:10.1111/j.1349-7006.2012.02216.x
41. Rolland P, Madjd Z, Durrant L, Ellis IO, Layfield R, Spendlove I. The ubiquitin-binding protein p62 is expressed in breast cancers showing features of aggressive disease. *Endocr Relat Cancer* (2007) 14(1):73–80. doi:10.1677/erc.1.01312
42. Luo RZ, Yuan ZY, Li M, Xi SY, Fu J, He J. Accumulation of p62 is associated with poor prognosis in patients with triple-negative breast cancer. *Onco Targets Ther* (2013) 6:883–8. doi:10.2147/OTT.S46222
43. Todoric J, Antonucci L, Di Caro G, Li N, Wu X, Lytle NK, et al. Stress-activated NRF2-MDM2 cascade controls neoplastic progression in pancreas. *Cancer Cell* (2017) 32(6):824–839.e828. doi:10.1016/j.ccell.2017.10.011
44. Kitamura H, Torigoe T, Asanuma H, Hisasue SI, Suzuki K, Tsukamoto T, et al. Cytosolic overexpression of p62 sequestosome 1 in neoplastic prostate tissue. *Histopathology* (2006) 48(2):157–61. doi:10.1111/j.1365-2559.2005.02313.x
45. Giatromanolaki A, Sivridis E, Mendrinos S, Koutsopoulos AV, Koukourakis MI. Autophagy proteins in prostate cancer: relation with anaerobic metabolism and Gleason score. *Urol Oncol* (2014) 32(1):39.e11–8. doi:10.1016/j.urolonc.2013.04.003
46. Kuo WL, Sharifi MN, Lingen MW, Ahmed O, Liu J, Nagilla M, et al. p62/SQSTM1 accumulation in squamous cell carcinoma of head and neck predicts sensitivity to phosphatidylinositol 3-kinase pathway inhibitors. *PLoS One* (2014) 9(3):e90171. doi:10.1371/journal.pone.0090171
47. Arai A, Chano T, Ikebuchi K, Hama Y, Ochi Y, Tameno H, et al. p62/SQSTM1 levels predicts radiotherapy resistance in hypopharyngeal carcinomas. *Am J Cancer Res* (2017) 7(4):881–91.
48. Iwadate R, Inoue J, Tsuda H, Takano M, Furuya K, Hirasawa A, et al. High expression of SQSTM1/p62 protein is associated with poor prognosis in epithelial ovarian cancer. *Acta Histochem Cytochem* (2014) 47(6):295–301. doi:10.1267/ahc.14048
49. Yan XY, Zhang Y, Zhang JJ, Zhang LC, Liu YN, Wu Y, et al. p62/SQSTM1 as an oncotarget mediates cisplatin resistance through activating RIP1-NF-kappaB pathway in human ovarian cancer cells. *Cancer Sci* (2017) 108(7):1405–13. doi:10.1111/cas.13276
50. Inui T, Chano T, Takikita-Suzuki M, Nishikawa M, Yamamoto G, Okabe H. Association of p62/SQSTM1 excess and oral carcinogenesis. *PLoS One* (2013) 8(9):e74398. doi:10.1371/journal.pone.0074398
51. Liu JL, Chen FF, Lung J, Lo CH, Lee FH, Lu YC, et al. Prognostic significance of p62/SQSTM1 subcellular localization and LC3B in oral squamous cell carcinoma. *Br J Cancer* (2014) 111(5):944–54. doi:10.1038/bjc.2014.355
52. Nakayama S, Karasawa H, Suzuki T, Yabuuchi S, Takagi K, Aizawa T, et al. p62/sequestosome 1 in human colorectal carcinoma as a potent prognostic predictor associated with cell proliferation. *Cancer Med* (2017) 6(6):1264–74. doi:10.1002/cam4.1093
53. Niklaus M, Adams O, Berezowska S, Zlobec I, Graber F, Slotta-Huspenina J, et al. Expression analysis of LC3B and p62 indicates intact activated autophagy is associated with an unfavorable prognosis in colon cancer. *Oncotarget* (2017) 8(33):54604–15. doi:10.18632/oncotarget.17554
54. Iwadate R, Inoue J, Tsuda H, Takano M, Furuya K, Hirasawa A, et al. High expression of p62 protein is associated with poor prognosis and aggressive phenotypes in endometrial cancer. *Am J Pathol* (2015) 185(9):2523–33. doi:10.1016/j.ajpath.2015.05.008
55. Ellis RA, Horswell S, Ness T, Lumsdon J, Tooze SA, Kirkham N, et al. Prognostic impact of p62 expression in cutaneous malignant melanoma. *J Invest Dermatol* (2014) 134(5):1476–8. doi:10.1038/jid.2013.497
56. Sample A, Zhao B, Qiang L, He YY. Adaptor protein p62 promotes skin tumor growth and metastasis and is induced by UVA radiation. *J Biol Chem* (2017) 292(36):14786–95. doi:10.1074/jbc.M117.786160
57. Cao QH, Liu F, Yang ZL, Fu XH, Yang ZH, Liu Q, et al. Prognostic value of autophagy related proteins ULK1, Beclin 1, ATG3, ATG5, ATG7, ATG9, ATG10, ATG12, LC3B and p62/SQSTM1 in gastric cancer. *Am J Transl Res* (2016) 8(9):3831–47.
58. Cancer Genome Atlas Research Network. Comprehensive genomic characterization of squamous cell lung cancers. *Nature* (2012) 489(7417):519–25. doi:10.1038/nature11404
59. Sporn MB, Liby KT. NRF2 and cancer: the good, the bad and the importance of context. *Nat Rev Cancer* (2012) 12(8):564–71. doi:10.1038/nrc3278
60. Totoki Y, Tatsuno K, Covington KR, Ueda H, Creighton CJ, Kato M, et al. Trans-ancestry mutational landscape of hepatocellular carcinoma genomes. *Nat Genet* (2014) 46(12):1267–73. doi:10.1038/ng.3126
61. Mitsuishi Y, Taguchi K, Kawatani Y, Shibata T, Nukiwa T, Aburatani H, et al. Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. *Cancer Cell* (2012) 22(1):66–79. doi:10.1016/j.ccr.2012.05.016
62. Umemura A, He F, Taniguchi K, Nakagawa H, Yamachika S, Font-Burgada J, et al. p62, upregulated during preneoplasia, induces hepatocellular carcinogenesis by maintaining survival of stressed HCC-initiating cells. *Cancer Cell* (2016) 29(6):935–48. doi:10.1016/j.ccell.2016.04.006
63. Amaravadi R, Kimmelman AC, White E. Recent insights into the function of autophagy in cancer. *Genes Dev* (2016) 30(17):1913–30. doi:10.1101/gad.287524.116
64. Park JS, Oh SY, Lee DH, Lee YS, Sung SH, Ji HW, et al. p62/SQSTM1 is required for the protection against endoplasmic reticulum stress-induced apoptotic cell death. *Free Radic Res* (2016) 50(12):1408–21. doi:10.1080/10715762.2016.1253073
65. Ji LL, Sheng YC, Zheng ZY, Shi L, Wang ZT. The involvement of p62-Keap1-Nrf2 antioxidative signaling pathway and JNK in the protection of natural flavonoid quercetin against hepatotoxicity. *Free Radic Biol Med* (2015) 85:12–23. doi:10.1016/j.freeradbiomed.2015.03.035

66. Su X, Li T, Liu Z, Huang Q, Liao K, Ren R, et al. Licochalcone A activates Keap1-Nrf2 signaling to suppress arthritis via phosphorylation of p62 at serine 349. *Free Radic Biol Med* (2018) 115:471–83. doi:10.1016/j.freeradbiomed.2017.12.004
67. Mizunoe Y, Kobayashi M, Sudo Y, Watanabe S, Yasukawa H, Natori D, et al. Trehalose protects against oxidative stress by regulating the Keap1-Nrf2 and autophagy pathways. *Redox Biol* (2018) 15:115–24. doi:10.1016/j.redox.2017.09.007
68. Kageyama S, Saito T, Obata M, Koide RH, Ichimura Y, Komatsu M. Negative regulation of the Keap1-Nrf2 pathway by a p62/Sqstm1 splicing variant. *Mol Cell Biol* (2018) 38(7):e00642-17. doi:10.1128/MCB.00642-17

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

MINI REVIEW
published: 16 March 2018
doi: 10.3389/fonc.2018.00073

Ataxia-Telangiectasia Mutated Kinase in the Control of Oxidative Stress, Mitochondria, and Autophagy in Cancer: A Maestro With a Large Orchestra

Venturina Stagni[1,2], Claudia Cirotti[1,2] and Daniela Barilà[1,2]**

[1] Department of Biology, University of Rome "Tor Vergata", Rome, Italy, [2] Laboratory of Cell Signaling, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Fondazione Santa Lucia, Rome, Italy

Ataxia-telangiectasia mutated kinase (ATM) plays a central role in the DNA damage response (DDR) and mutations in its gene lead to the development of a rare autosomic genetic disorder, ataxia telangiectasia (A-T) characterized by neurodegeneration, premature aging, defects in the immune response, and higher incidence of lymphoma development. The ability of ATM to control genome stability several pointed to ATM as tumor suppressor gene. Growing evidence clearly support a significant role of ATM, in addition to its master ability to control the DDR, as principle modulator of oxidative stress response and mitochondrial homeostasis, as well as in the regulation of autophagy, hypoxia, and cancer stem cell survival. Consistently, A-T is strongly characterized by aberrant oxidative stress, significant inability to remove damaged organelles such as mitochondria. These findings raise the question whether ATM may contribute to a more general hijack of signaling networks in cancer, therefore, playing a dual role in this context. Indeed, an unexpected tumorigenic role for ATM, in particular, tumor contexts has been demonstrated. Genetic inactivation of Beclin-1, an autophagy regulator, significantly reverses mitochondrial abnormalities and tumor development in ATM-null mice, independently of DDR. Furthermore, ATM sustains cancer stem cells survival by promoting the autophagic flux and ATM kinase activity is enhanced in HER2-dependent tumors. This mini-review aims to shed new light on the complexity of these new molecular circuits through which ATM may modulate cancer progression and to highlight a novel role of ATM in the control of proteostasis.

Keywords: ataxia-telangiectasia mutated kinase, oxidative stress, mitophagy/autophagy, cancer, proteostasis

OPEN ACCESS

Edited by:
Giuseppe Filomeni, Danish Cancer Society, Denmark

Reviewed by:
Apolinar Maya-Mendoza, Danish Cancer Society, Denmark
Shiaw-Yih Lin, University of Texas MD Anderson Cancer Center, United States

****Correspondence:***
Venturina Stagni
venturina.stagni@gmail.com;
Daniela Barilà
daniela.barila@uniroma2.it

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *01 February 2018*
Accepted: *02 March 2018*
Published: *16 March 2018*

Citation:
Stagni V, Cirotti C and Barilà D (2018) Ataxia-Telangiectasia Mutated Kinase in the Control of Oxidative Stress, Mitochondria, and Autophagy in Cancer: A Maestro With a Large Orchestra. Front. Oncol. 8:73. doi: 10.3389/fonc.2018.00073

INTRODUCTION

Redox Homeostasis

Reactive oxygen species (ROS) are physiologically by-products of cellular metabolism and play a central role in many physiological and pathological processes including inflammation and chronic diseases such as atherosclerosis and cancer, underscoring the importance of investigating cellular pathways involved in redox homeostasis (1, 2).

Main sources of ROS are enzymes and organelles such as mitochondria (3). About 2–4% of oxygen consumed by mitochondrial oxidative phosphorylation is partially reduced and flows through membranes to activate signaling pathways that have then to be promptly turned off. Intracellular enzymatic and non-enzymatic antioxidant defense is responsible for redox homeostasis, preventing ROS accumulation (4). Together with ROS, reactive nitrogen species (RNS) are harmful molecules mostly generated by spontaneous reaction between ROS and nitric oxide signaling molecule (5).

Reactive oxygen species and RNS damage proteins as well as cellular organelles; therefore, several systems evolved to regulate and preserve a functional cellular protein pool, to ensure the quality and functionality of cellular organelles, and to finally guarantee the maintenance of proteostasis (3, 6). The autophagy-lysosomal machinery (7), the ubiquitin–proteasomal system (8), and molecular chaperones, including heat shock proteins (HSPs) (9, 10), cooperate to this aim and, indeed, they are all finely regulated by oxidative stress, which augments their functionality in order to support proteostasis and organelle quality control in challenging conditions (6).

Ataxia-Telangiectasia Mutated Kinase (ATM) and Oxidative Stress Response

Ataxia-telangiectasia mutated kinase is a serine/threonine protein kinase, and it is a well-characterized tumor suppressor gene, which plays a central role in the nucleus in the DNA damage response (DDR). In humans, loss of function in ATM results in ataxia telangiectasia (A-T), a pleiotropic disease whose hallmarks include neurodegeneration, cancer-proneness, premature aging, radio-sensitivity, metabolic, and immune dysfunctions (11). For many years, the defect in DNA-damage response has been considered the solely responsible for A-T phenotype.

Increasing numbers of reports have described elevated readouts of oxidative stress in plasma of A-T patients, in cultured A-T fibroblasts and lymphocytes, and in tissues and cultured cells from Atm-deficient mice (12, 13). Notably, the response of A-T fibroblasts to induced oxidative stress was found defective [reviewed in Ref. (14)].

Consistently with the loss of redox homeostasis, mitochondria, are severely compromised in A-T appearing swollen and with disrupted cristae structure; as a consequence, A-T cells display mitochondrial ROS overproduction and decreased ATP levels (15). Interestingly, some of the pathological phenotypes identified in A-T, including insulin resistance, premature aging, and neurodegeneration cannot be easily connected to the well-known role of ATM in DDR, while conversely, they could be linked to the interplay between ATM and ROS (16, 17). More importantly, the administration of antioxidants to $Atm^{-/-}$ mice ameliorates the disease progression and delayed cancer development (thymic lymphomas), by reducing ROS and restoring mitochondrial membrane potential (18).

These observations were at first puzzling and, more recently, they could be linked to a role of ATM in regulating cellular oxidative stress signaling. In particular, ATM is activated in the cytosol by ROS through the formation of ATM dimers *via* disulfide bonds (16, 19). Downstream to oxidative stress-dependent activation, ATM regulates a number of processes to promote restoration of redox homeostasis including adjustment of glutathione levels and activation of pentose phosphate pathway (20), regulation of mitochondrial mass, function and turnover (15, 21, 22), removal of peroxisomes *via* autophagy (23). More recently, ATM activation in response to oxidative stress has been shown to be involved in the control of proteostasis, preventing protein aggregation through a still unknown mechanism (24).

ATM AND AUTOPHAGY

The autophagy system is a finely regulated catabolic process responsible for the selective removal of cytoplasmic components (i.e., proteins, aggregates, or whole organelles) properly targeted by posttranslational modifications (ubiquitination). Basal autophagy physiologically occurs to ensure proteins turnover, maintaining intracellular homeostasis. Moreover, the autophagy system is activated by oxidative stress triggered by endogenous and exogenous stressors including nutrient starvation, hypoxia, and mitochondria and peroxisome dysfunction (25).

Ataxia-telangiectasia mutated kinase is activated in the cytosol by all the conditions listed above (16, 26); moreover, it has a role in autophagy induction (22, 27). It has been clearly demonstrated that ATM sustains autophagic pathway by inhibiting the negative regulator mTOR complex 1 (mTORC1). At the molecular level, ATM activation upon oxidative and/or nitrosative stress is responsible for the activation of LKB1/AMPK/TSC2 signaling axis, culminating with mTORC1 inhibition and relieving its repression on ULK1, which is the key protein responsible for the nucleation and formation of the autophagosome membrane, further activated by AMPK-mediated phosphorylation. This signaling pathway starting from ATM culminates in autophagy flux induction (22, 27).

The same pathway is also activated by ATM upon ROS induction under hypoxia (28). In this context, ATM promotes HIF1a stabilization by direct phosphorylation on Ser696, culminating on mTORC1 inhibition (28). Consistently, under hypoxic conditions, ATM-deficient cells fail to activate HIF1a and to inhibit mTORC1, further supporting the requirement for ATM in this pathway (28). Evidence for a role of ATM in the modulation of HIF-1a basal expression has also been provided (29, 30).

Finally, a recent work suggested that ATM regulates autophagy also by sustaining the levels and activity of ATG4C protease in cancer cells grown as mammospheres (31), characterized by low ROS levels (32). Interestingly, ATG4 proteases are the only ATG members that act as oxidative stress sensors (33). It has been demonstrated that oxidative signal leads to inactivation of ATG4s by oxidation of essential cystein residues on these proteins, at the site of autophagosome formation, thereby promoting lipidation of ATG8, an essential step in the process of autophagy (33). These data suggest that the ATM–ATG4C axis may represent a new molecular link that connects ROS, ATM, and autophagy signaling (31).

Overall, these publications suggest a role of ATM in the cytosol in regulating autophagosome formation upon exogenous and endogenous oxidative stress.

ATM IN SELECTIVE AUTOPHAGY: MITOPHAGY AND PEXOPHAGY

The main source of intracellular ROS are metabolically active organelles, such as mitochondria and peroxisomes (34, 35). Not surprisingly, ATM localizes to both these compartments to sense ROS increase and to activate pro-survival or pro-death intracellular pathways, depending on the intensity of the stimuli (15, 23, 36). The role of ATM in preserving mitochondrial functionality is well documented since many years. *In vivo,* loss of ATM results in mitochondria abnormalities causing ROS overproduction, strong decrease in ATP levels, and ultrastructural alterations. Moreover, the selective removal of damaged mitochondria, process known as mitophagy, is strongly impaired causing the accumulation of dysfunctional organelles (15). More recently, these evidences have been recapitulated also in neuroblastoma cells: ATM depletion results in a similar mitochondrial phenotype and mitophagy alteration, partially rescued by NAD+ cofactor replenishment (37). Taken together, these papers demonstrate the relation between ATM and mitochondria.

Although the molecular mechanism responsible for ATM function in the control of mitochondrial homeostasis deserves further investigation, it has been demonstrated that ATM activation upon mitochondrial stress or ROS increase protects cells from damage; indeed, ATM-mediated modulation of the well-characterized PINK1–Parkin pathway promotes the elimination *via* mitophagy of altered mitochondria (38).

Very recently, ATM localization to peroxisomes and its role in peroxisomes selective removal, named pexophagy, has been described. As for mitochondria, ATM localizes to peroxisomes probably to sense ROS increase and prevent damage. ATM localization in peroxisomes outer membrane is mediated by its interaction with PEX5, a peroxisome import receptor. Upon peroxisomal ROS increase, ATM-mediated PEX5 phosphorylation targets PEX5 for mono-ubiquitination and recognition by autophagic-adaptor protein (such as p62), incorporating dysfunctional organelles into autophagic vescicles (23). Very interestingly, pexophagy defects observed in ATM-deficient cells are rescued by reconstitution of ATM expression, confirming the direct role of ATM in this response (23).

The removal of damaged organelles described, so far, is also sustained by ATM-dependent induction of general autophagy, as ATM inhibits the autophagy negative regulator mTORC1, sustaining ULK1 pro-autophagic protein activation as described above (22).

Taken all together, these evidences highlight a relevant role of ATM in the cytosol: ATM ensures a prompt reply to ROS increase by activating autophagy, mitophagy, and pexophagy in order to preserve proteostasis and cellular homeostasis.

ROS-DEPENDENT ATM ACTIVATION AND CANCER

Elevated rates of ROS have been detected in almost all cancers, where they promote many aspects of tumor development and progression (39, 40). In cancer cells, high levels of ROS can result from increased metabolic activity, mitochondrial dysfunction, peroxisome activity, increased cellular receptor signaling, oncogene activity, increased activity of oxidases, cyclooxygenases, lipoxygenases, and thymidine phosphorylase, or through crosstalk with infiltrating immune cells (41). Moreover, ROS deregulation in low oxygen tension or hypoxia condition is a common feature of all solid tumors, it is strongly associated with tumor development, malignant progression, metastatic outgrowth, and resistance to therapy and it is considered an independent prognostic indicator for poor patient prognosis in various tumor types (42). It has been largely demonstrated that ROS increase leads to proteome oxidation and instability, and alteration of the proteostasis control machine (9). More interestingly, in order to survive under stress conditions (i.e., ROS increase/hypoxia condition/starvation), many cancer cells adapt their proteostasis network and become uniquely dependent on it, an example of non-oncogene addiction (43). Individual nodes of the proteostasis network, such as Hsp90 and other HSP chaperones involved in the protein quality control networks, are currently exploited as drug targets in cancer and entered in clinical trials (44, 45).

The identification of new cytoplasmic signaling mediated by ATM in response to oxidative stress (46) and the finding that ATM can regulate networks that ensure proteins and organelles quality open the question whether these networks may contribute to A-T pathogenesis and to cancer progression (16).

It has been hypothesized for a long time that higher cancer predisposition of A-T patients depends exclusively on defects in ATM-dependent-DDR, which leads to genomic instability (11). Unexpectedly, allelic loss of the autophagy regulator Beclin-1, significantly delayed tumor development in ATM-null mice. This effect was not associated to the rescue of DNA damage signaling but rather to a significant reversal of the mitochondrial abnormalities (15). Accordingly, it has been also demonstrated that Rapamycin (mTOR inhibitor) and antioxidant treatments rescue ATM-dependent lymphomagenesis, suggesting that the dysregulation of mTORC1 and ROS contribute to A-T pathology (22). Moreover, suppression of ATM may significantly contribute to the activation of mTORC1 observed in hypoxic tumors and can promote tumor cell survival through autophagy regulation (28). Importantly, autophagy is a dichotomous phenomenon, involved in cell growth as well as in cell death, depending on its magnitude and on the cell context (47). Autophagy, as DNA damage, has been proposed to play a tumor-suppressive role in the early stages of tumorigenesis and, indeed, it is upregulated by several tumor suppressor genes; however, above a certain threshold, autophagy can also induce cell death and, if triggered appropriately, can be used as a means of killing cancer cells (48). Paradoxically, it was recently published that autophagy promotes the stem-like phenotype in breast cancer, suggesting a controversial role in cancer of autophagy (49). Interestingly, it has been reported that ATG4A and Beclin1 autophagic genes are upregulated in breast cancer stem cells (BCSCs) and are essential genes involved in BCSCs formation and maintenance (50, 51). Overall, these papers support the idea that BCSCs utilize autophagy for survival and growth, suggesting that, in this context, autophagy promotes tumor progression and tumor relapse acting as a tumor-promoting signaling. Interestingly, it was recently demonstrated that ATM kinase

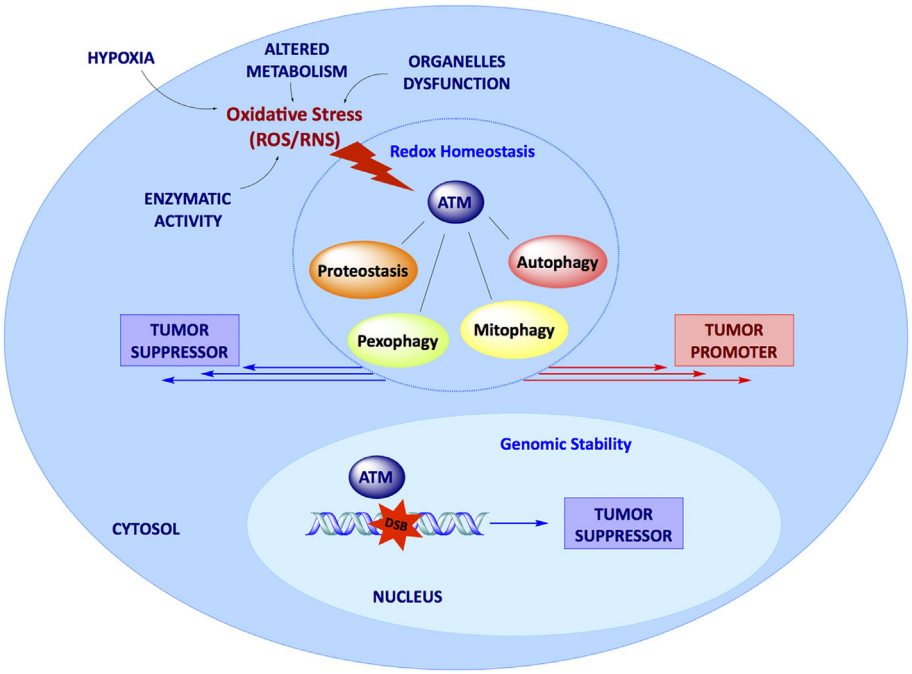


FIGURE 1 | Dual role of ataxia-telangiectasia mutated kinase (ATM) in cancer. In the nucleus, DBSs activate ATM kinase, which ensures genomic stability, acting as a tumor suppressor factor. In the cytosol, ATM acts as a stress sensor, being activated upon oxidative stress to maintain intracellular redox homeostasis. Here, ATM is responsible for protein quality control and regulates several pathways such as autophagy and organelles selective removal (mitophagy and pexophagy). All these pathways may promote or prevent tumor growth depending on the specific context; the molecular mechanisms underlying the dual function of ATM still deserve further elucidation.

could have a pro-survival role in BCSCs through regulation of ATG4C gene and autophagy (31).

Finally, the expression of HSP90, a central player in the control of proteostasis, increases under stress conditions (as ROS accumulation upon oxidative stress) and, it is exploited by cancer cells to support the stability and the aberrant activity of oncoproteins overexpressed or mutated in malignancy including HER2, BCR-ABL, and EGFR (45, 52). According to this observation, HSP90 is one of the most actively pursued cancer drug targets and several different HSP90 inhibitors entered in clinical trials so far (52). Growing evidences support the idea that ATM could regulate HSP90 activity. ATM kinase can directly phosphorylate HSP90 (53, 54) although the significance of these posttranslational modifications is still largely unknown. More interestingly, we recently demonstrated that ATM activity sustains HSP90 interaction with its client protein HER2, promoting its stabilization and, therefore, sustaining HER2-dependent tumorigenicity (55, 56). These data suggest a new connection between ATM kinase and HSP90 chaperone: ATM may contribute to the control of protein quality and stability and could also modulate tumor progression *via* the regulation of this heat shock protein.

CONCLUSION

In conclusion, although the canonical role of ATM in the management of DNA damage defines ATM as a tumor suppressor gene, the identification of several novel functions of ATM, mostly related to its activation in response to oxidative stress and to its ability to modulate the cellular response to this insult, support multiple roles of ATM in cancer (**Figure 1**). ATM-dependent regulation of autophagy, mitophagy, pexophagy, and proteostasis suggest the idea that the effect of ATM expression and activity in cancer may be the result of its multiple functions in several signaling pathways and may, therefore, be strictly dependent on the specific cellular context. More studies are urgently needed to ascertain the molecular mechanisms through which this panel of cytosolic functions of ATM could modulate cancer development and therapy.

AUTHOR CONTRIBUTIONS

VS and DB came up with the topic for this mini-review; VS, DB, and CC wrote and edited the text.

FUNDING

This work has been supported by research grants to Associazione Italiana per la Ricerca sul Cancro-AIRC (IG8706), by Ministero dell'Istruzione, dell'Università e della Ricerca, JPI HDHL NUTRICOG MiTyrAge, and PRIN_2015LZE9944_005 to DB; VS has been supported by AIRC (IG8706) and CC by JPI HDHL NUTRICOG MiTyrAge project.

REFERENCES

1. Jones DP. Redefining oxidative stress. *Antioxid Redox Signal* (2006) 10:1865–79. doi:10.1089/ars.2006.8.1865
2. Poprac P, Jomova K, Simunkova M, Kollar V, Rhodes CJ, Valko M. Targeting free radicals in oxidative stress-related human diseases. *Pharmacol Sci* (2017) 38:592–607. doi:10.1016/j.tips.2017.04.005
3. Holmström KM, Finkel T. Cellular mechanisms and physiological consequences of redox-dependent signalling. *Nat Rev Mol Cell Biol* (2014) 15: 411–21. doi:10.1038/nrm3801
4. Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O. Oxidative stress and antioxidant defense. *World Allergy Organ J* (2012) 5:9–19. doi:10.1097/WOX.0b013e3182439613
5. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* (2007) 87:245–313. doi:10.1152/physrev.00044.2005
6. Reichmann D, Voth W, Jakob U. Maintaining a healthy proteome during oxidative stress. *Mol Cell* (2018) 69:203–13. doi:10.1016/j.molcel.2017.12.021
7. Liang Y, Sigrist S. Autophagy and proteostasis in the control of synapse aging and disease. *Curr Opin Neurobiol* (2017) 48:113–21. doi:10.1016/j.conb.2017.12.006
8. Ciechanover A, Kwon TK. Protein quality control by molecular chaperones in neurodegeneration. *Front Neurosci* (2017) 11:185. doi:10.3389/fnins.2017.00185
9. Niforou K, Cheimonidou C, Trougakos IP. Molecular chaperones and proteostasis regulation during redox imbalance. *Redox Biol* (2014) 2:323–32. doi:10.1016/j.redox.2014.01.017
10. Hetz C, Papa FR. The unfolded protein response and cell fate control. *Mol Cell* (2018) 69:169–81. doi:10.1016/j.molcel.2017.06.017
11. Shiloh Y, Ziv Y. The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. *Nat Rev Mol Cell Biol* (2013) 14:197–210. doi:10.1038/nrm3546
12. Kamsler A, Daily D, Hochman A, Stern N, Shiloh Y, Rotman G, et al. Increased oxidative stress in ataxia telangiectasia evidenced by alterations in redox state of brains from Atm-deficient mice. *Cancer Res* (2001) 61:1849–54.
13. Reichenbach J, Schubert R, Schindler D, Müller K, Böhles H, Zielen S. Elevated oxidative stress in patients with ataxia telangiectasia. *Antioxid Redox Signal* (2002) 4:465–9. doi:10.1089/15230860260196254
14. Shiloh Y, Lederman HM. Ataxia-telangiectasia (A-T): an emerging dimension of premature ageing. *Ageing Res Rev* (2017) 33:76–88. doi:10.1016/j.arr.2016.05.002
15. Valentin-Vega YA, MacLean KH, Tait-Mulder J, Milasta S, Steeves M, Dorsey FC, et al. Mitochondrial dysfunction in ataxia-telangiectasia. *Blood* (2012) 119:1490–500. doi:10.1182/blood-2011-08-373639
16. Ditch S, Paull TT. The ATM protein kinase and cellular redox signaling: beyond the DNA damage response. *Trends Biochem Sci* (2012) 37:15–22. doi:10.1016/j.tibs.2011.10.002
17. Choy KR, Watters DJ. Neurodegeneration in ataxia-telangiectasia: multiple roles of ATM kinase in cellular homeostasis. *Dev Dyn* (2018) 247:33–46. doi:10.1002/dvdy.24522
18. Schubert R, Erker L, Barlow C, Yakushiji H, Larson D, Russo A, et al. Cancer chemoprevention by the antioxidant tempol in Atm-deficient mice. *Hum Mol Genet* (2004) 13:1793–802. doi:10.1093/hmg/ddh189
19. Guo Z, Deshpande R, Paull TT. ATM activation in the presence of oxidative stress. *Cell Cycle* (2010) 9:4805–11. doi:10.4161/cc.9.24.14323
20. Cosentino C, Grieco D, Costanzo A. ATM activates the pentose phosphate pathway promoting anti-oxidant defence and DNA repair. *EMBO J* (2011) 30:546–55. doi:10.1038/emboj.2010.330
21. Ambrose M, Goldstine JV, Gatti RA. Intrinsic mitochondrial dysfunction in ATM-deficient lymphoblastoid cells. *Hum Mol Genet* (2007) 16:2154–64. doi:10.1093/hmg/ddm166
22. Alexander A, Cai S-L, Kim J, Nanez A, Sahin M, MacLean KH, et al. ATM signals to TSC2 in the cytoplasm to regulate mTORC1 in response to ROS. *Proc Natl Acad Sci U S A* (2010) 107:4153–8. doi:10.1073/pnas.0913860107
23. Zhang J, Tripathi DN, Jing J, Alexander A, Kim J, Powell RT, et al. ATM functions at the peroxisome to induce pexophagy in response to ROS. *Nat Cell Biol* (2015) 17:1259–69. doi:10.1038/ncb3230
24. Lee JH, Mand MR, Kao CH, Zhou Y, Ryu SW, Richards AL, et al. ATM directs DNA damage responses and proteostasis via genetically separable pathways. *Sci Signal* (2018) 11:eaan5598. doi:10.1126/scisignal.aan5598
25. Kroemer G, Mariño G, Levine B. Autophagy and the integrated stressresponse. *Mol Cell* (2010) 40: 280-93. doi:10.1016/j.molcel.2010.09.023
26. Bhatti S, Kozlov S, Farooqi AA, Naqui A, Lavin MF, Khanna KK. ATM protein kinase: the linchpin of cellular defenses to stress. *Cell Mol Life Sci* (2011) 68:2977–3006. doi:10.1007/s00018-011-0683-9
27. Tripathi DN, Chowdhury R, Trudel LJ, Tee AR, Slack RS, Walker CL, et al. Reactive nitrogen species regulate autophagy through ATM-AMPK-TSC2-mediated suppression of mTORC1. *Proc Natl Acad Sci U S A* (2013) 110:E2950–7. doi:10.1073/pnas.1307736110
28. Cam H, Easton J, High A, Houghton P. mTORC1 signaling under hypoxic conditions is controlled by ATM-dependent phosphorylation of HIF-1α. *Mol Cell* (2010) 40:509–20. doi:10.1016/j.molcel.2010.10.030
29. Ousset M, Bouquet F, Fallone F, Biard D, Dray C, Vlet P, et al. Loss of ATM positively regulates the expression of hypoxia inducible factor 1 (HIF-1) through oxidative stress: role in the physiopathology of the disease. *Cell Cycle* (2010) 9:2814–22. doi:10.4161/cc.9.14.12253
30. Mongiardi P, Stagni V, Natoli M, Giaccari D, D'Agnano I, Falchetti M, et al. Oxygen sensing is impaired in ATM defective cells. *Cell Cycle* (2011) 10:4311–20. doi:10.4161/cc.10.24.18663
31. Antonelli M, Strappazzon F, Arisi I, Brandi R, D'Onofrio M, Sambucci M, et al. ATM kinase sustains breast cancer stem-like cells by promoting ATG4C expression and autophagy. *Oncotarget* (2017) 8:21692–709. doi:10.18632/oncotarget.15537
32. Wu T, Harder BG, Wong PK, Lang JE, Zhang DD. Oxidative stress, mammospheres and Nrf2-new implication for breast cancer therapy? *Mol Carcinog* (2015) 54:1494–502. doi:10.1002/mc.22202
33. Scherz-Shouval R, Shvets E, Fass E, Shorer H, Gil L, Elazar Z. Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. *EMBO J* (2007) 26:1749–60. doi:10.1038/sj.emboj.7601623
34. Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J* (2009) 417:1–13. doi:10.1042/BJ20081386
35. Schrader M, Fahimi HD. Peroxisomes and oxidative stress. *Biochim Biophys Acta* (2006) 1763:1755–66. doi:10.1016/j.bbamcr.2006.09.006
36. Watters D, Kedar P, Spring K, Bjorkman J, Chen P, Gatei M, et al. Localization of a portion of extranuclear ATM to peroxisomes. *J Biol Chem* (1999) 274:34277–82. doi:10.1074/jbc.274.48.34277
37. Fang EF, Kassahun H, Croteau DL, Scheibye-Knudsen M, Marosi K, Lu H, et al. NAD+ replenishment improves lifespan and healthspan in ataxia telangiectasia models via mitophagy and DNA repair. *Cell Metab* (2016) 24:566–81. doi:10.1016/j.cmet.2016.09.004
38. Qi Y, Qiu Q, Gu X, Tian Y, Zhang Y. ATM mediates spermidine-induced mitophagy via PINK1 and Parkin regulation in human fibroblasts. *Sci Rep* (2016) 6:24700. doi:10.1038/srep24700
39. Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nat Rev Drug Discov* (2009) 8:579–91. doi:10.1038/nrd2803
40. Panieri E, Santoro MM. ROS homeostasis and metabolism: a dangerous liason in cancer cells. *Cell Death Dis* (2016) 7:e2253. doi:10.1038/cddis.2016.105
41. Gào X, Schöttker B. Reduction-oxidation pathways involved in cancer development: a systematic review of literature reviews. *Oncotarget* (2017) 8:51888–906. doi:10.18632/oncotarget.17128
42. Liou GY, Storz P. Reactive oxygen species in cancer. *Free Radic Res* (2010) 44:479–96. doi:10.3109/10715761003667554
43. Deshaies RJ. Proteotoxic crisis, the ubiquitin-proteasome system, and cancer therapy. *BMC Biol* (2014) 12:94. doi:10.1186/s12915-014-0094-0
44. Rappa F, Farina F, Zummo G, David S, Campanella C, Carini F, et al. HSP-molecular chaperones in cancer biogenesis and tumor therapy: an overview. *Anticancer Res* (2012) 32:5139–50.
45. Neckers L, Workman P. Hsp90 molecular chaperone inhibitors: are we there yet? *Clin Cancer Res* (2012) 18:64–76. doi:10.1158/1078-0432.CCR-11-1000
46. Guo Z, Kozlov S, Lavin MF, Person MD, Paull TT. ATM activation by oxidative stress. *Science* (2010) 330:517–21. doi:10.1126/science.1192912
47. Galluzzi L, Pietrocola F, Bravo-San Pedro JM, Amaravadi RK, Baehrecke EH, Cecconi F, et al. Autophagy in malignant transformation and cancer progression. *EMBO J* (2015) 34:856–80. doi:10.15252/embj.201490784

48. Rodolfo C, Di Bartolomeo S, Cecconi F. Autophagy in stem and progenitor cells. *Cell Mol Life Sci* (2016) 73:475–96. doi:10.1007/s00018-015-2071-3
49. Maycotte P, Jones KL, Goodall ML, Thorburn J, Thorburn A. Autophagy supports breast cancer stem cell maintenance by regulating IL6 secretion. *Mol Cancer Res* (2015) 13:651–8. doi:10.1158/1541-7786.MCR-14-0487
50. Wolf J, Dewi DL, Fredebohm J, Müller-Decker K, Flechtenmacher C, Hoheisel JD, et al. A mammosphere formation RNAi screen reveals that ATG4A promotes a breast cancer stem-like phenotype. *Cancer Res* (2013) 15:R109. doi:10.1186/bcr3576
51. Gong C, Bauvy C, Tonelli G, Yue W, Deloménie C, Nicolas V, et al. Beclin 1 and autophagy are required for the tumorigenicity of breast cancer stem-like/progenitor cells. *Oncogene* (2013) 32:2261–72. doi:10.1038/onc.2012.252
52. Jhaveri K, Ochiana SO, Dunphy MP, Gerecitano JF, Corben AD, Peter RI, et al. Heat shock protein 90 inhibitors in the treatment of cancer: current status and future directions. *Expert Opin Investig Drugs* (2014) 23:611–28. doi:10.1517/13543784.2014.902442
53. Matsuoka S, Ballif B, Smogorzewska A, McDonald E 3rd, Hurov K, Luo JL, et al. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* (2007) 316:1160–6. doi:10.1126/science.1140321
54. Elaimy AL, Ahsan A, Marsh K, Pratt WB, Ray D, Lawrence TS, et al. ATM is the primary kinase responsible for phosphorylation of Hsp90α after ionizing radiation. *Oncotarget* (2016) 7:82450–7. doi:10.18632/oncotarget.12557
55. Stagni V, Manni I, Oropallo V, Mottolese M, Di Benedetto A, Piaggio G, et al. ATM kinase sustains HER2 tumorigenicity in breast cancer. *Nat Commun* (2015) 16(6):6886. doi:10.1038/ncomms7886
56. Stagni V, Oropallo V, Barilà D. ATM: an unexpected tumor-promoting factor in HER2-expressing tumors. *Mol Cell Oncol* (2015) 3:e1054551. doi:10.1080/23723556.2015.1054551

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer AM-M and the handling editor declared their shared affiliation.

frontiers in Oncology

ORIGINAL RESEARCH
published: 04 June 2018
doi: 10.3389/fonc.2018.00195

Involvement of NADPH Oxidase 1 in Liver Kinase B1-Mediated Effects on Tumor Angiogenesis and Growth

*Elisabetta Zulato[1], Francesco Ciccarese[2], Giorgia Nardo[1], Marica Pinazza[1], Valentina Agnusdei[1], Micol Silic-Benussi[1], Vincenzo Ciminale[1,2] and Stefano Indraccolo[1]**

[1] *Istituto Oncologico Veneto IOV – IRCCS, Padova, Italy,* [2] *Department of Surgery, Oncology and Gastroenterology, University of Padova, Padova, Italy*

The liver kinase B1 (*LKB1*) gene is a tumor suppressor with an established role in the control of cell metabolism and oxidative stress. However, whether dis-regulated oxidative stress promotes growth of LKB1-deficient tumors remains substantially unknown. Through *in vitro* studies, we observed that loss of LKB1 perturbed expression of several genes involved in reactive oxygen species (ROS) homeostasis. In particular, this analysis evidenced strongly up-modulated NADPH oxidase 1 (*NOX1*) transcript levels in tumor cells lacking LKB1. NOX1 accounted in part for enhanced cytotoxic effects of H_2O_2-induced oxidative stress in A549 LKB1-deficient tumor cells. Notably, genetic and pharmacologic inhibition of NOX1 activity reduced angiogenesis and growth of A549 tumors in mice. These results suggest that NOX1 inhibitors could counteract ROS production and the angiogenic switch in LKB1-deficient tumors.

Keywords: NADPH oxidase 1, liver kinase B1, angiogenesis, lung cancer, reactive oxygen species, oxidative stress

OPEN ACCESS

Edited by:
Giuseppe Filomeni, Danish Cancer Society, Denmark

Reviewed by:
Alfredo Criollo, Universidad de Chile, Chile
Zhi-Xiang Xu, University of Alabama at Birmingham, United States

****Correspondence:***
Stefano Indraccolo
stefano.indraccolo@unipd.it

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *09 February 2018*
Accepted: *14 May 2018*
Published: *04 June 2018*

Citation:
Zulato E, Ciccarese F, Nardo G, Pinazza M, Agnusdei V, Silic-Benussi M, Ciminale V and Indraccolo S (2018) Involvement of NADPH Oxidase 1 in Liver Kinase B1-Mediated Effects on Tumor Angiogenesis and Growth. Front. Oncol. 8:195. doi: 10.3389/fonc.2018.00195

INTRODUCTION

Increased oxidative stress is common in cancer cells. In fact, uncontrolled proliferation is connected with profound metabolic remodeling accompanied by altered redox status. Reactive oxygen species (ROS) are important biological messengers involved in cell signaling, tumor growth, and metastasis (1). Recent studies suggest that increased oxidative stress could be exploited for therapeutic purposes (2, 3), as tumor cells might be particularly vulnerable to drugs which further increase oxidative stress or otherwise block biochemical buffers of ROS (4).

The liver kinase B1 (LKB1) is a serine threonine kinase, whose hereditable mutations are associated with the Peutz-Jeghers syndrome and is somatically mutated or deleted in certain tumor types, with relatively high frequencies in non-small cell lung cancer (NSCLC) and cervical carcinoma (5). LKB1 is involved in energy homeostasis control, as well as control of cell proliferation and polarity (6). LKB1 and its downstream effector AMP-activated protein kinase (AMPK) have also been recently involved in metabolic adaptation and in the regulation of tumor cell response to oxidative stress by various mechanisms including control of NADPH homeostasis (7) and enhancement of the activity of superoxide dismutase-2 and catalase by a p38-mediated mechanism (8). These studies largely utilized LKB1-deficient tumor cell lines and their matched LKB1-proficient counterpart to

Abbreviations: LKB1, liver kinase B1; NOX1, NADPH oxidase 1; ROS, reactive oxygen species; NSCLC, non-small cell lung cancer; AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; CCLE, cancer cell line encyclopedia; NAC, *N*-acetyl cysteine; MVD, microvessel density; NRF2, nuclear respiratory factor 2; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

investigate effects of this kinase on oxidative stress. Although several features and mechanisms associated with altered ROS homeostasis in LKB1-mutant tumor cells have been uncovered by using this experimental system, previous studies did not analyze the transcriptional perturbations of genes involved in redox control in LKB1-deficient cell lines.

Here, we investigated at transcriptional level three pairs of LKB1-deficient cell lines and observed that LKB1 is involved in the regulation of NADPH oxidase 1 (*NOX1*) transcript expression. Inhibition of NOX1 activity attenuated cytotoxic effects of oxidative stress in LKB1-deficient tumor cells *in vitro* and was an effective strategy to prevent the angiogenic switch and growth of LKB1-deficient A549 tumors *in vivo*. Although marked heterogeneity in the expression levels of *NOX1* among LKB1-deficient lung and cervical cancer cell lines does not allow us to generalize these findings, our results hint at NOX1 as a candidate mediator of angiogenesis in a subset of LKB1-deficient tumors.

MATERIALS AND METHODS

Cell Culture and *In Vitro* Treatments

A549 cells were provided by Dr. Reuben J. Shaw (Salk Institute for Biological Studies, La Jolla, CA, USA). H460, H1650, H1975, HeLa, SiHa, and HEK293T cells were purchased from ATCC (Manassas, VA, USA). H23, H1437, H2228, H2009, and H1299 cell lines were kindly provided by Dr. Massimo Broggini (Mario Negri Institute, Milan, Italy). HeLa, SiHa, and HEK293T cells were cultured in Dulbecco modified Eagle medium (DMEM; EuroClone, Milan, Italy), supplemented with 10% FCS (EuroClone), 10 mM HEPES, and 1% antibiotic–antimycotic mix (Thermo Fisher, Waltham, MA, USA). H460, H1650, H1975, H23, H1437, H2228, H2009, and H1299 cells were grown in RPMI1640 medium (EuroClone) supplemented with 10% FCS, 1% HEPES, 1% Na pyruvate, 2 mM L-Glutamine (Lonza, Basel, Switzerland), and 1% antibiotic–antimycotic mix. A549 cells were cultured in DMEM-F12 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% FCS, 2 mM L-Glutamine, and 1% antibiotic–antimycotic mix. In some experiments, cell lines were treated with the appropriate vehicle or with the following substances: 2 mM hydrogen peroxide (H_2O_2, Sigma-Aldrich, Saint Luis, MO, USA), 2 mM *N*-acetylcysteine (NAC, Sigma-Aldrich), 100 μM apocynin (Sigma-Aldrich), for the indicated time points.

Retrovirus Production and Transduction With Viral Particles

The retroviral vector overexpressing human LKB1^{wt} (RV-LKB1^{wt}; plasmid # 8592) and the corresponding control vector (pBABE, plasmid # 1764) used to transduce LKB1-mutated cells were purchased from Addgene (Cambridge, MA, USA). Viral particles were produced by transfecting HEK293T cells with second-generation packaging plasmids. Virus was harvested 24 and 48 h after transfection and concentrated by ultracentrifugation at 24,000 rpm for 2 h. A549, H460, and HeLa cells were transduced with a VSV-G-pseudotyped retroviral vector encoding LKB1 cDNA (LKB1^{wt}) or with an empty vector (pBABE). Briefly, 0.5×10^6 cells were incubated at 37°C overnight and 200 μl of concentrated vector-containing supernatant were layered over target cells. The day after, cells were washed and cultured in fresh medium. Cells expressing the constructs were selected in puromycin-containing medium (8 μg/ml for A549 and H460 cells, 4 μg/ml for HeLa cells) for 10–14 days prior to subsequent analysis.

Reverse Transcription-PCR and Quantitative PCR

Total RNA was isolated using RNAeasy® Mini Kit (Qiagen, Venlo, Netherlands) according to manufacturer's instructions. cDNA was synthesized from 0.3 to 1 μg of total RNA using High Capacity RNA-to-cDNA Kit (Thermo Fisher). Expression levels of 20 oxidative metabolism-related genes were analyzed by real-time Ready custom panels (Roche Diagnostics, Basel, Switzerland). Real-time PCRs were performed using Platinum® SYBR® Green (Thermo Fisher) in an ABI Prism 7900 HT Sequence Detection System (Thermo Fisher). Results were analyzed using the ΔΔCt method with normalization against HMBS gene expression.

Genes included in Custom Panels were selected based on their role in oxidative metabolism and are reported in **Table 1**. Primers used for qRT-PCR are reported in **Table 2**.

TABLE 1 | Genes included in custom panel.

Gene ID	Description	HGNC ID	Pathway
GCLC	*Glutamate-cysteine ligase, catalytic subunit*	4311	
GCLM	*Glutamate-cysteine ligase, modifier subunit*	4312	Glutathione synthesis
GSS	*Glutathione synthetase*	4624	
GGT6	*Gamma-glutamyltransferase 6*	26891	Glutathione degradation
GGT7	*Gamma-glutamyltransferase 7*	4259	
NFE2L2	*Nuclear factor erythroid 2-like 2; nuclear respiratory factor 2*	7782	Transcription factors involved in glutathione metabolism
JUN	*jun proto-oncogene*	6204	
ABCC1	*ATP-binding cassette sub-family C member 1*	51	Glutathione adducts transporters
ABCC2	*ATP-binding cassette sub-family C member 2*	53	
CAT	*Catalase*	1516	Enzymes involved in antioxidant defenses
TXN	*Thioredoxin*	12435	
SOD2	*Superoxide dismutase-2*	11180	
GPX1	*Glutathione peroxidase 1*	4353	
GSR	*Glutathione reductase*	4623	
MSRA	*Methionine sulfoxide reductase A*	7377	
NOX1	*NADPH oxidase 1*	7889	Enzymes involved in reactive oxygen species production
CBS	*Cystathionine β-synthase*	1550	Enzymes involved in transsulfuration pathway
CTH	*Cystathionine γ-lyase*	2505	
SLC7A11	*Solute carrier family 7 member 11; xCT*	11059	Cystine and cysteine transporters
SLC7A10	*Solute carrier family 7 member 10; ASC1*	11058	
HMBS	*Hydroxymethylbilane synthase*	4982	Reference gene
B2M	*Beta-2-microglobulin*	914	
ACTB	*Actin, beta*	132	

TABLE 2 | Primers used for qRT-PCR.

Gene ID	Forward primer	Reverse primer
NADPH oxidase 1	5′-GGGGTCAAACAGAG GAGAGC-3′	5′-CCACTTCCAAGACT CAGGGG-3′
HMBS	5′-GGCAATGCGGCTGCAA-3′	5′-GGGTACCCACGCGA ATCAC-3′

TABLE 3 | Stealth siRNAs used in transfection experiments.

Stealth siRNA name	Stealth siRNA code	Sense sequence
siRNA	Negative Control Low GC Duplex	AGCUACACUAUCGAGCAAUUAACUU
siNOX1	NOX1HSS178287	UGGGAUGAUCGUGACUCCCACUGUA

Annexin-V/Propidium Iodide Assay

Cells were incubated with Annexin-V-FITC in Hepes buffer containing propidium iodide (PI), using the Annexin-V Fluos Staining Kit (Roche Diagnostics, Mannheim, Germany). Labeled cells were analyzed on EPICS-XL cytofluorimeter using Expo32 software (Beckman Coulter, Fullerton, CA, USA). Dot plot images were obtained by using the Kalusa analysis 1.3 software (Beckman Coulter).

Measurement of Mitochondrial Membrane Potential ($\Delta\psi$)

Cells were incubated with tissue culture medium containing 15 nM tetramethylrhodamine, methyl ester (TMRM; Thermo Fisher) and 20 ng/ml Verapamil (efflux pumps inhibitor; Sigma-Aldrich) for 15 min at room temperature. Labeled cells were analyzed with an LSRII flow cytometer using the 560 nm laser and the 585/15 nm filter.

RNAi Experiments

Cells (3×10^5) were seeded in six-well tissue culture plates. The following day, Opti-MEM® I Medium with GlutaMAX™ (Thermo Fisher) was mixed separately with 20 nM Stealth RNAi™ siRNA (Thermo Fisher) and with Lipofectamine® RNAiMAX Transfection Reagent (Thermo Fisher) and incubated 5 min at room temperature. Separated mix were combined and, following additional 20 min of incubation, 500 µl of final mix were added, drop by drop, to adherent cells, along with 1.5 ml of culture medium. Lipofection was blocked after 6 h, through removal of medium with liposomes and addition of culture medium. Sequences of Stealth RNAi™ siRNAs are reported in **Table 3**.

In Vivo Experiments

Procedures involving animals and their care conformed to institutional guidelines that comply with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 12 December, 1987) and were authorized by the ethical committee of the University of Padova and the Italian Ministry of Health (Authorization no. 143/2012-B). For tumor establishment, both dorsolateral flanks of 8-week-old SCID mice (Charles River, Wilmington, MA, USA) were injected subcutaneously with 0.5×10^6 LKB1^{mut} A549 tumor cells mixed at 4°C with liquid Matrigel (Becton-Dickinson). Tumor volume (mm^3) was measured by caliber and calculated according to the following formula: $L \times l^2 \times 0.5$, where L is the longest diameter, l is the shortest diameter, and 0.5 is a constant to calculate the volume of an ellipsoid. In the experiment with NAC, LKB1^{mut} A549 cells were 16 h pretreated with NAC 2 mM before injection and during tumor growth NAC was administered intraperitoneally at 150 mg/kg every day. Control mice received intraperitoneal injections of PBS. In the experiment in which expression level of *NOX1* was reduced by siRNA-mediated silencing, LKB1^{mut} A549 cells were injected 48 h after lipofection. In the experiment with apocynin, LKB1^{mut} A549 cells were 16 h pretreated with apocynin at 100 µM before injection and during tumor growth apocynin was administered *via* oral gavage at 16 mg/kg every day.

At the end of the experiment, mice were killed by cervical dislocation. For all experiments, five to six mice per group were used. The tumors were harvested by dissection and snap-frozen.

Immunofluorescence Analysis (IFA)

Tumor vessels were labeled with a rat anti-CD31 mAb (1:50 dilution; Becton-Dickinson) followed by staining with a goat anti-rat 546 secondary antibody (Thermo Fisher). Sections of 5 µm were cut from frozen biopsies and microvessel density (MVD) was quantified by screening the CD31-stained sections for the areas of highest vascularity, as previously reported (9). Briefly, nuclei were stained with TO-PRO-3 Iodide (1:1,000 dilution; Thermo Fisher). Immunofluorescence signals were visualized on a Zeiss Axiovert 100 M confocal microscope (Carl Zeiss AG; Oberkochen, Germany), using argon (488 nm) and helium-neon (543–633 nm) laser sources. For each sample, the number of fields analyzed varied between 5 and 10 per sample, depending on the size of sections; at least 4 samples per group were analyzed. Images were collected at a magnification of 200×. MVD was quantified by screening for the areas of highest vascularity. Two independent operators blindly evaluated the images: morphologically identifiable vessels were counted as an individual vessel.

Statistical Analysis

Results were expressed as mean value ± SD. Student's *t*-test or Mann–Whitney test were used depending on the distribution of values. Differences were considered statistically significant when $P < 0.05$. For each cell line, data are expressed as fold change arbitrarily setting controls at 1. If not specified otherwise, the symbol § was used for comparison between LKB1^{mut} and LKB1^{wt} cells, the symbol * for comparison between treated and untreated cells and the symbol $^{\#}$ for comparison between treated cells and cells pre-treated with other drugs (NAC, Apocynin) or siRNA.

RESULTS

LKB1 Regulates Expression of Genes Involved in ROS Metabolism in Tumor Cells

To investigate whether LKB1 modulated expression of genes involved in the response to cellular redox stress, we compared

expression levels of 20 relevant genes (**Table 1**) by real-time PCR in human tumor cell lines isogenic for LKB1 *status* and grown *in vitro* under standard conditions. We performed these experiments using the LKB1-deficient NSCLC cell lines A549 and H460, and cervical carcinoma cell line HeLa transduced with a control retroviral vector (hereafter referred to as LKB1^{mut}) and their LKB1-proficient counterparts obtained by retroviral transduction of wild-type LKB1 cDNA (hereafter referred to as LKB1^{wt}). As expected, LKB1 reconstitution fully restored AMPK and acetyl-CoA carboxylase phosphorylation under glucose starvation—a condition known to trigger LKB1-dependent AMPK activation and associated with oxidative stress [(10) and data not shown]. Remarkably, in all cell lines tested, several antioxidant genes were downregulated and others were upregulated following reconstitution of LKB1 (**Figure 1**). Most of these variations were, however, not shared by the various cell lines analyzed. The most consistently downregulated gene in LKB1^{wt} cells was *NOX1*, a homolog of the macrophage *NOX2*, accounting for the respiratory burst in response to pathogens (11). NADPH oxidases catalyze the transfer of one electron from NADPH to oxygen, generating superoxide or H_2O_2, thus further increasing oxidative stress (12).

Further analysis of *NOX1* mRNA levels in nine NSCLC and two cervical cancer cell lines, with known *LKB1* mutational status, showed that almost all cell lines bearing *LKB1* mutations presented increased *NOX1* expression compared with *LKB1* wild-type cells (**Figure 2A**). However, broad heterogeneity in *NOX1* expression was detected in LKB1-mutant cell lines, being *NOX1* levels particularly high in A549 cells (**Figure 2A**). An additional *in silico* analysis was performed by matching the *LKB1* mutational status and *NOX1* mRNA transcript data of tumor cell lines, available at the COSMIC and Cancer Cell Line Encyclopedia web sites, respectively. Results showed that *NOX1* transcripts were significantly increased in LKB1-mutated NSCLC cell lines, thus strengthening our observations (**Figure 2B**). The relatively high expression of *NOX1* in A549 cells led us to focus on these cells to investigate the possible involvement of NOX1 in the regulation of oxidative stress.

NOX1 Accounts for Increased Sensitivity to Exogenous Oxidative Stress in A549 Cells

Gene silencing and pharmacologic inhibition approaches were used to analyze whether NOX1 could account for increased sensitivity to oxidative stress in LKB1^{mut} cells. Interestingly, siRNA-mediated *NOX1* silencing in A549 cells increased resistance of LKB1^{mut} A549 cells to H_2O_2-induced cell death, while leaving substantially unaffected response to oxidative stress in LKB1^{wt} cells (**Figure 3A**). Consistent with this, measurement of mitochondrial membrane potential showed a significant increase in the number of TMRM negative LKB1^{mut} A549 cells upon H_2O_2-treatment (**Figure 3B**). This phenomenon in the mitochondrial membrane potential was partially rescued by *NOX1* silencing. Similar results were obtained following NOX1 inhibition with apocynin (**Figure 3C**). It should be noted that despite interference with NOX1 activity, LKB1^{mut} cells showed increased H_2O_2-induced cell death compared with LKB1^{wt} cells (**Figures 3A–C**), probably

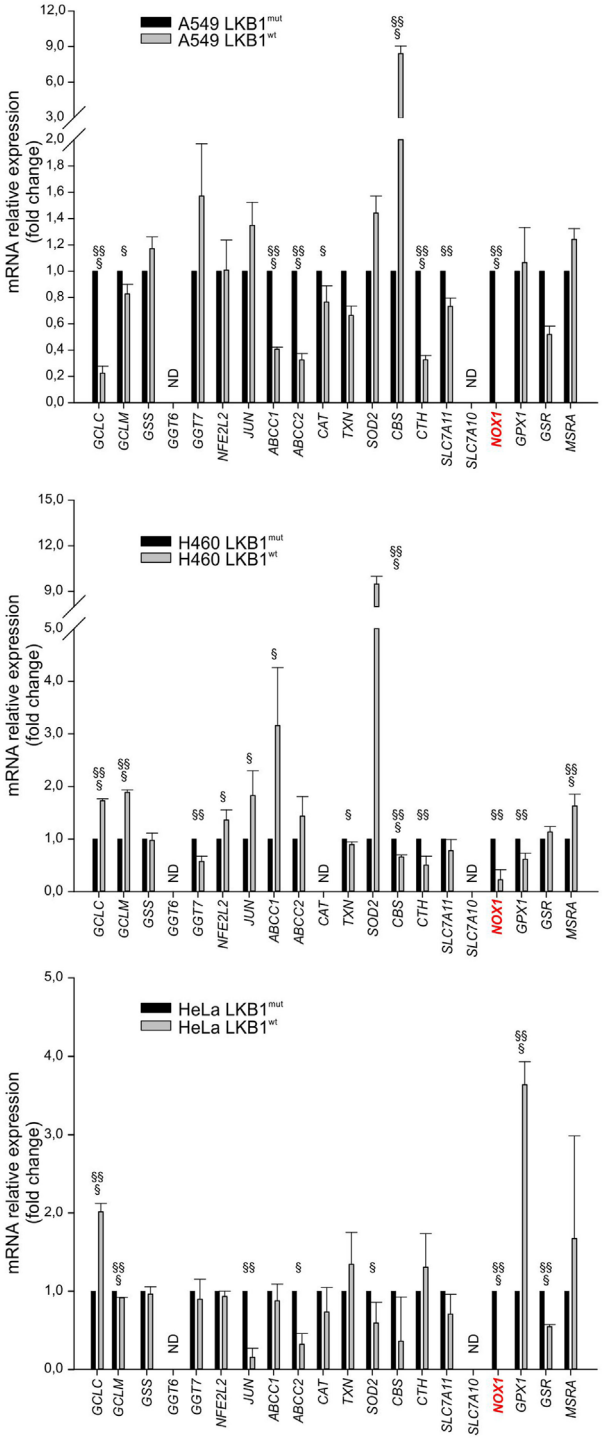
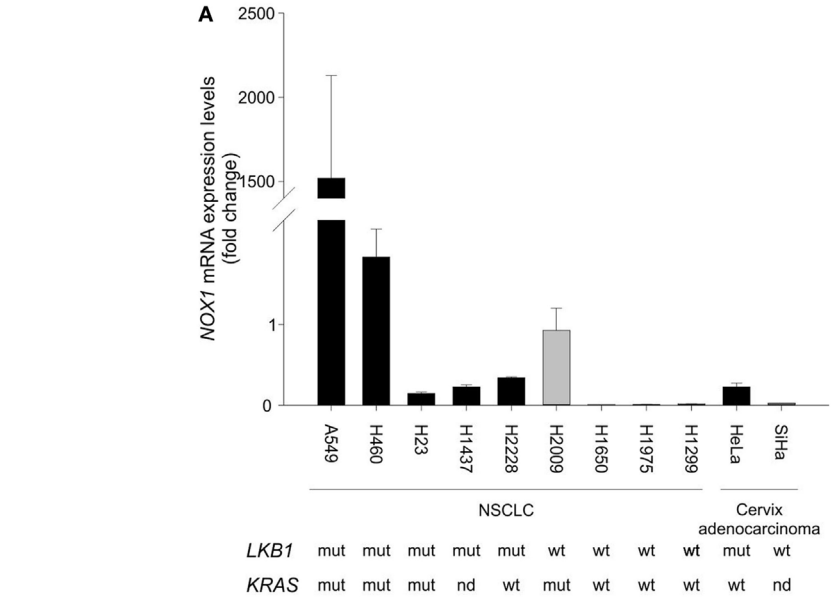
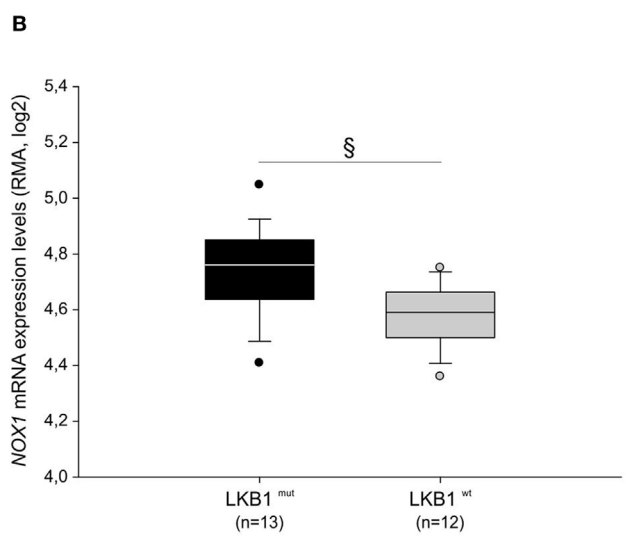


FIGURE 1 | Liver kinase B1 (LKB1) *status* affects expression of oxidative metabolism-related genes. Expression profile of selected genes in isogenic pairs of A549 (top), H460 (middle), and HeLa (bottom) cells. Transcription levels of each gene were quantified by real-time Ready Custom Panels and normalized to *HMBS* mRNA content. Results are reported as mean value ± SD of three independent experiments and are expressed as fold change with respect to LKB1^{mut} cells, arbitrary set as 1 for each analyzed transcript ($^{§}P < 0.05$, $^{§§}P < 0.01$, $^{§§§}P < 0.001$). ND = expression level not determined.





LKB1 mut	LKB1 wt
HCC15	NCI H522
HCC44	NCI H1299
NCI H23	HCC4006
A549	NCI H1650
NCI H1395	NCI H1781
NCIH460	NCI H2087
LU65	NCI H2009
NCI H1184	HCC2279
NCI H2126	NCI H3255
HCC366	NCI H1975
VMRCLCD	CALU1
NCI H2122	NCI H2171
NCI H1437	

FIGURE 2 | Liver kinase B1 (LKB1) controls NADPH oxidase 1 (*NOX1*) expression in human cell lines. *NOX1* expression levels in LKB1-deficient (LKB1^{mut}) and LKB1-proficient (LKB1^{wt}) non-small cell lung cancer (NSCLC) and cervical cancer cell lines. **(A)** Expression levels of *NOX1* mRNA in NSCLC and cervical cancer cell lines, with defined *LKB1* and *KRAS* mutational *status*. Results are reported as mean value ± SD of three independent experiments and are expressed as fold change compared to H2009 cells, arbitrary set at 1. **(B)** *In silico* analysis of *NOX1* levels in NSCLC cell lines. Expression level of *NOX1* as Robust Multi-array Average (RMA) values (source: Cancer Cell Line Encyclopedia). The dispersion of *NOX1* expression values in $n = 13$ *LKB1* mutated and in $n = 12$ *LKB1* wild-type cancer cell lines is represented as box-plots, indicating the first and the third quartiles and the median. The list of *LKB1*-mutated and non-mutated cancer cell lines is reported in the table ($^{§}P < 0.05$). nd = mutational status not determined.

due to either incomplete blockade of NOX1 or multifactorial mechanisms of control of oxidative stress by LKB1.

NOX1 Promotes Angiogenesis and Growth of LKB1-Deficient A549 Tumors

High levels of ROS have been involved in induction of angiogenesis and tumor growth (13). To investigate whether oxidative stress could play a role in LKB1^{mut} tumor formation, we co-injected LKB1^{mut} A549 cells along with administration of the antioxidant NAC. LKB1^{mut} A549 cells formed tumors (**Figure 4A**), and co-injection with NAC resulted in significant reduction of tumor growth and reduction of tumor vascularization, assessed 7 days after tumor cell injection (**Figure 4A**). Notably, chronic administration of apocynin significantly reduced tumor growth and MVD of LKB1^{mut} tumors (**Figure 4A**), suggesting that blockade of NOX

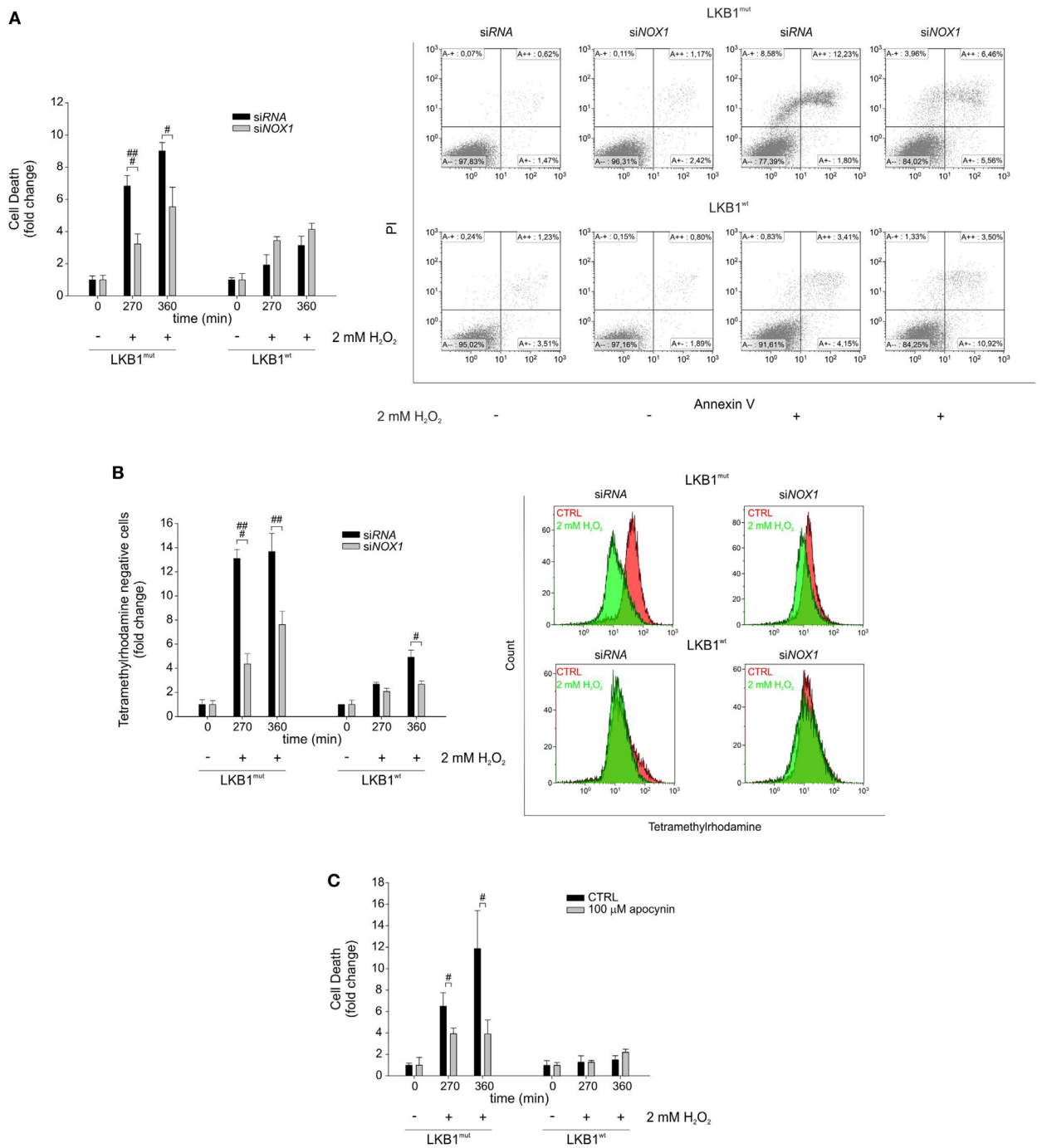


FIGURE 3 | NADPH oxidase 1 (NOX1) increases sensitivity to exogenous oxidative stress *in vitro*. **(A)** A549 LKB1^{mut} and LKB1^{wt} cells were transiently transfected with si*RNA* targeting *NOX1* and cell death was measured upon H_2O_2 treatment (only statistically significant differences between silenced and control cells are indicated, $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$). A representative dot plot diagram of Annexin-V/PI stained cells is shown (right panel). **(B)** Measurement of mitochondrial membrane potential in A549 LKB1^{mut} and LKB1^{wt} cells transiently silenced for *NOX1* expression (only statistically significant differences between silenced and control cells are indicated, $^{\#}P < 0.05$, $^{\#\#}P < 0.001$, $^{\#\#\#}P < 0.001$). **(C)** Induction of cell death in A549 LKB1^{mut} or LKB1^{wt} cells treated with the NOX pharmacological inhibitor apocynin 100 µM upon H_2O_2 treatment (only statistically significant differences between apocynin-treated cells and control cells are indicated, $^{\#}P < 0.05$).

activity impairs angiogenesis and tumor growth. These results were confirmed and strengthened by siRNA-mediated silencing of *NOX1* in A549 cells prior to their injection in mice (**Figure 4B**). Analysis of expression levels of *NOX1* in samples recovered 7 days after tumor cell injection showed, as expected, marginal silencing of the target gene, in contrast to the >80% reduction of *NOX1*

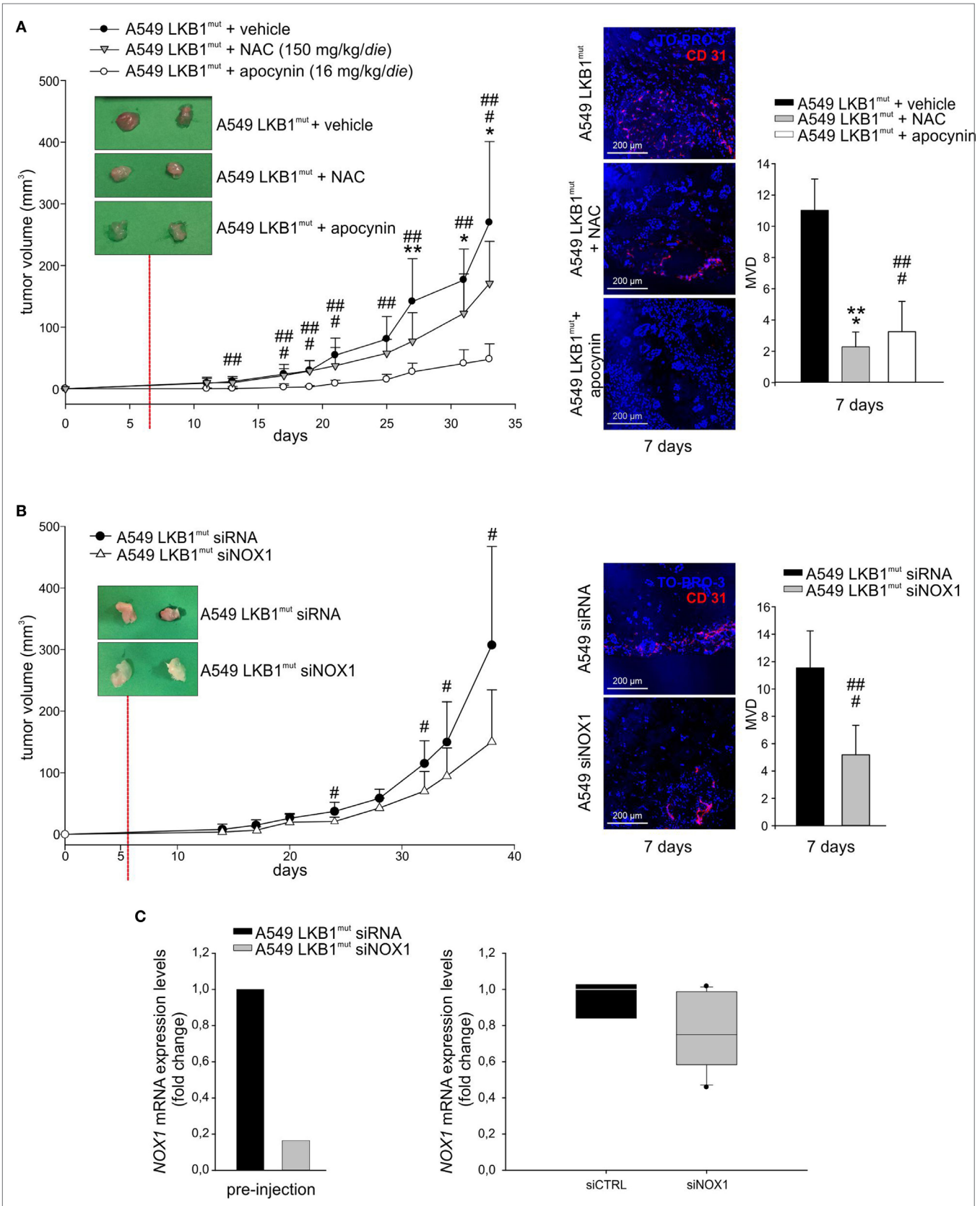


FIGURE 4 | Continued

FIGURE 4 | NADPH oxidase 1 (NOX1) impacts on tumor growth and angiogenesis *in vivo*. **(A)** A549 LKB1^{mut} cells were s.c. injected in SCID mice and treated daily with either *N*-acetyl cysteine (NAC) (150 mg/kg/day, gray triangles), or apocynin (16 mg/kg/day, white dots), or vehicle (black dots) (left panel). The image of two representative masses/group shows a difference in vascularization of treated compared to untreated tumors 7 days after tumor cells injection, as indicated by CD31 staining (magnification 200×) and quantification of the microvessel density (MVD) (right panel) (n = 3–6 fields from five samples/group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ A549 LKB1^{mut} NAC-treated versus untreated tumors; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ A549 LKB1^{mut} apocynin-treated versus untreated tumors). **(B)** Growth curves of s.c. tumors originated from A549 LKB1^{mut} cells transiently transfected with si*RNA* control (black dots) or with a si*RNA* targeting *NOX1* (white triangles) (left panel). Seven days after tumor cells injection, LKB1^{mut} si*RNA* tumors appear more vascularized compared to LKB1^{mut} si*NOX1* tumors, as shown by the representative image. Immunofluorescence analysis of CD31^{+} cells and quantification of microvasculature in tumors at day 7 (right panel) (n = 3–6 fields from five samples/group; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ A549 LKB1^{mut} si*RNA* versus si*NOX1*). **(C)** Expression levels of *NOX1* in LKB1-deficient (LKB1^{mut}) cells were analyzed prior to their injection in mice (bottom panel, at left) and in samples obtained from experiment shown in B (n = 3 and n = 6 for si*RNA* and si*NOX1* samples, respectively) and recovered 1 week post injection (bottom panel, at right).

levels at time of injection (**Figure 4C**). This suggests that NOX1 could play a key role in the initial phases of the angiogenic switch in the A549 LKB1^{mut} tumor xenograft model.

DISCUSSION

This study disclosed for the first time an effect of LKB1 on *NOX1* expression in tumor cells. According to our results, LKB1 acts as a suppressor of *NOX1* expression at the RNA level. Among a set of 20 genes involved in redox homeostasis, *NOX1* transcript was consistently down-regulated following forced expression of LKB1 cDNA in LKB1-deficient cancer cells. Other genes were also perturbed, presumably as a direct consequence of LKB1 loss or, alternatively, as adaptive response to compensate for loss of protective mechanisms. In this respect, Kaufman et al., reported that LKB1 loss leads to activation of the nuclear respiratory factor 2 transcription factor, which regulates oxidative stress response genes (14).

NADPH oxidase 1 belongs to a family of NADPH oxidase (11) and cancer cells generally express more NOX isoforms. In the case of A549 cells utilized in our study, *NOX2* was expressed at a much lower baseline level compared to *NOX1* and *NOX4* was not expressed (data not shown). Therefore, although apocynin is not an isoform-selective NOX inhibitor (11), effects measured following treatment of A549 cells with this compound are likely to be accounted for by NOX1. A second consideration regards *NOX1* levels in tumor cells. In fact, real-time PCR experiments showed marked differences in the relative expression levels of *NOX1* among the NSCLC and cervical cancer cell lines analyzed (**Figure 2A**). It can, therefore, be speculated that the biological consequences of NOX1 inhibition might also depend on the amount of NOX1 in tumor cells. In line with this hypothesis, we indeed observed marked reduction of H_2O_2-induced cell death following apocynin treatment of A549 cells but not HeLa cells, which express substantially less NOX1 transcripts (**Figure 2** and data not shown). Therefore, although LKB1 acted as suppressor of *NOX1* expression in all cell lines analyzed, other endogenous factors are likely to modulate *NOX1* expression in tumor cells. According to previous studies, one of these factors could be KRAS, as its activating mutations were shown to correlate with *NOX1* overexpression in human colon cancer samples (15) and in cancer cells (16). In this regard, analysis of NSCLC cancer cell lines showed increased *NOX1* mRNA levels in cell lines bearing *LKB1* and *KRAS* mutations (**Figure 2**). This heterogeneity may also have precluded the inclusion of NOX1 among top ranking genes regulated by LKB1 in other genome-wide studies which previously investigated transcriptional profiles in LKB1-mutant cells and clinical samples (5, 14, 17, 18).

Although a detailed explanation of the mechanism connecting LKB1 to NOX1 is lacking and goes beyond the aim of our study, some hypothesis can be advanced. First, although LKB1 is not a transcription factor it is localized both in the cytoplasm and the nucleus of cells (17, 19). Therefore, suppression of NOX1 by LKB1 could be accounted for by its still poorly characterized nuclear functions. In this regard, it has been demonstrated that LKB1 forms a nuclear complex with the transcription regulatory factor LMO4, the transcription factor GATA-6 and Ldb1, and it has been suggested that this complex is involved in the regulation of GATA-mediated gene expression (20). Intriguingly, GATA-binding sites have been identified within the *NOX1* promoter (21). It is thus possible that LKB1 could be involved in the regulation of *NOX1* mRNA transcript through a GATA-6 mediated mechanism. Second, among indirect mechanisms, it has been shown that loss of LKB1 especially combined with KRAS mutations (as is the case in A549 cells) triggers the serine–glycine-one-carbon pathway, leading to increased S-adenosylmethionine generation, increased activity of DNA methyltransferases and elevation in DNA methylation (22). Hypothetically, this might silence some unknown repressor and thus unleash *NOX1* expression. Hence, alterations of NOX1 expression might occur as consequence of changes in epigenetic marks following vector-mediated re-expression of LKB1 in tumor cells. Further studies are required to validate these hypothesis.

The second innovative observation of this study regards the anti-angiogenic effects of drugs affecting ROS homeostasis in the context of LKB1-deficient tumors. Previous studies reported a link between angiogenesis and LKB1 expression in tumor models. Zhuang et al. described reduction of MVD accompanied by reduced expression of the angiogenic factors basic fibroblast growth factor and vascular endothelial growth factor (VEGF) in LKB1-deficient MDA-MB435 xenografts when LKB1 cDNA was added back, but further mechanistic insights were not provided (23). Along this line, it was known that NOX1 can promote the angiogenic switch in NIH-3T3 fibroblasts converting them into tumorigenic cells by a mechanism involving generation of ROS and increased expression of VEGF mRNA (24). Therefore, in view of our findings, it is conceivable that some of the effects on VEGF expression that follow interventions on LKB1 might depend on modulation of *NOX1* levels in tumor cells. Interestingly, Okon et al. reported that LKB1 functions as a

RAB7 effector and suppresses angiogenesis by promoting the cellular trafficking of neuropilin-1 from the RAB7 vesicles to the lysosomes for degradation (25). Neuropilin-1 is an angiogenic receptor that can be expressed both by endothelial and tumor cells (26). When expressed by tumor cells, neuropilin-1 seems to increase VEGF-mediated angiogenesis. Therefore, it can be speculated that adding LKB1 back to tumor cells triggers complex changes in the tumor microenvironment involving NOX1, ROS, and neuropilin-1 that impact on VEGF/VEGFR-2 signaling and angiogenesis with negative rebounds on tumor growth that largely exceed the modest effects on tumor cell proliferation, which can be detected *in vitro*. As the contribution of each component of this envisioned model could vary in individual tumors, additional studies are required to validate this hypothesis both in preclinical models and in patients.

In conclusion, our study disclosed for the first time that LKB1 controls *NOX1* expression *in vitro*. Perturbing ROS homeostasis or inhibition of NOX1 reduced tumor growth and angiogenesis *in vivo* and could represent a new possible therapeutic approach in LKB1-deficient KRAS-mutated tumors.

ETHICS STATEMENT

Procedures involving animals and their care conformed to institutional guidelines that comply with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 12 December, 1987) and were authorized by the ethical committee of the University of Padova and the Italian Ministry of Health (Authorization no. 143/2012-B).

AUTHOR CONTRIBUTIONS

SI conceived the study, designed experiments, analyzed data, and wrote the paper. EZ and FC designed and performed experiments, analyzed data, and wrote the paper. GN, MP, VA, and MS-B performed experiments and analyzed data. VC analyzed data and corrected the paper.

FUNDING

This work was supported by grants from the AIRC (IG18803 to SI).

REFERENCES

1. Panieri E, Santoro MM. ROS homeostasis and metabolism: a dangerous liason in cancer cells. *Cell Death Dis* (2016) 7:e2253. doi:10.1038/cddis.2016.105
2. Ciccarese F, Ciminale V. Escaping death: mitochondrial redox homeostasis in cancer cells. *Front Oncol* (2017) 7:117. doi:10.3389/fonc.2017.00117
3. Raza MH, Siraj S, Arshad A, Waheed U, Aldakheel F, Alduraywish S, et al. ROS-modulated therapeutic approaches in cancer treatment. *J Cancer Res Clin Oncol* (2017) 143:1789–809. doi:10.1007/s00432-017-2464-9
4. Gorrini C, Harris IS, Mak TW. Modulation of oxidative stress as an anticancer strategy. *Nat Rev Drug Discov* (2013) 12:931–47. doi:10.1038/nrd4002
5. Ji H, Ramsey MR, Hayes DN, Fan C, Mcnamara K, Kozlowski P, et al. LKB1 modulates lung cancer differentiation and metastasis. *Nature* (2007) 448:807–10. doi:10.1038/nature06030
6. Boudeau J, Sapkota G, Alessi DR. LKB1, a protein kinase regulating cell proliferation and polarity. *FEBS Lett* (2003) 546:159–65. doi:10.1016/S0014-5793(03)00642-2
7. Jeon SM, Chandel NS, Hay N. AMPK regulates NADPH homeostasis to promote tumour cell survival during energy stress. *Nature* (2012) 485:661–5. doi:10.1038/nature11066
8. Xu HG, Zhai YX, Chen J, Lu Y, Wang JW, Quan CS, et al. LKB1 reduces ROS-mediated cell damage via activation of p38. *Oncogene* (2015) 34:3848–59. doi:10.1038/onc.2014.315
9. Trenti A, Zulato E, Pasqualini L, Indraccolo S, Bolego C, Trevisi L. Therapeutic concentrations of digitoxin inhibit endothelial focal adhesion kinase and angiogenesis induced by different growth factors. *Br J Pharmacol* (2017) 174:3094–106. doi:10.1111/bph.13944
10. Spitz DR, Sim JE, Ridnour LA, Galoforo SS, Lee YJ. Glucose deprivation-induced oxidative stress in human tumor cells. A fundamental defect in metabolism? *Ann N Y Acad Sci* (2000) 899:349–62. doi:10.1111/j.1749-6632.2000.tb06199.x
11. Altenhofer S, Radermacher KA, Kleikers PW, Wingler K, Schmidt HH. Evolution of NADPH oxidase inhibitors: selectivity and mechanisms for target engagement. *Antioxid Redox Signal* (2015) 23:406–27. doi:10.1089/ars.2013.5814
12. Kamata T. Roles of Nox1 and other Nox isoforms in cancer development. *Cancer Sci* (2009) 100:1382–8. doi:10.1111/j.1349-7006.2009.01207.x
13. Xia C, Meng Q, Liu LZ, Rojanasakul Y, Wang XR, Jiang BH. Reactive oxygen species regulate angiogenesis and tumor growth through vascular endothelial growth factor. *Cancer Res* (2007) 67:10823–30. doi:10.1158/0008-5472.CAN-07-0783
14. Kaufman JM, Amann JM, Park K, Arasada RR, Li H, Shyr Y, et al. LKB1 loss induces characteristic patterns of gene expression in human tumors associated with NRF2 activation and attenuation of PI3K-AKT. *J Thorac Oncol* (2014) 9:794–804. doi:10.1097/JTO.0000000000000173
15. Laurent E, Mccoy JW III, Macina RA, Liu W, Cheng G, Robine S, et al. Nox1 is over-expressed in human colon cancers and correlates with activating mutations in K-Ras. *Int J Cancer* (2008) 123:100–7. doi:10.1002/ijc.23423
16. Park MT, Kim MJ, Suh Y, Kim RK, Kim H, Lim EJ, et al. Novel signaling axis for ROS generation during K-Ras-induced cellular transformation. *Cell Death Differ* (2014) 21:1185–97. doi:10.1038/cdd.2014.34
17. Jimenez AI, Fernandez P, Dominguez O, Dopazo A, Sanchez-Cespedes M. Growth and molecular profile of lung cancer cells expressing ectopic LKB1: down-regulation of the phosphatidylinositol 3'-phosphate kinase/PTEN pathway. *Cancer Res* (2003) 63:1382–8.
18. Lin-Marq N, Borel C, Antonarakis SE. Peutz-Jeghers LKB1 mutants fail to activate GSK-3beta, preventing it from inhibiting Wnt signaling. *Mol Genet Genomics* (2005) 273:184–96. doi:10.1007/s00438-005-1124-y
19. Nardo G, Favaro E, Curtarello M, Moserle L, Zulato E, Persano L, et al. Glycolytic phenotype and AMP kinase modify the pathologic response of tumor xenografts to VEGF neutralization. *Cancer Res* (2011) 71:4214–25. doi:10.1158/0008-5472.CAN-11-0242
20. Setogawa T, Shinozaki-Yabana S, Masuda T, Matsuura K, Akiyama T. The tumor suppressor LKB1 induces p21 expression in collaboration with LMO4, GATA-6, and Ldb1. *Biochem Biophys Res Commun* (2006) 343:1186–90. doi:10.1016/j.bbrc.2006.03.077
21. Adachi Y, Shibai Y, Mitsushita J, Shang WH, Hirose K, Kamata T. Oncogenic Ras upregulates NADPH oxidase 1 gene expression through MEK-ERK-dependent phosphorylation of GATA-6. *Oncogene* (2008) 27:4921–32. doi:10.1038/onc.2008.133
22. Kottakis F, Nicolay BN, Roumane A, Karnik R, Gu H, Nagle JM, et al. LKB1 loss links serine metabolism to DNA methylation and tumorigenesis. *Nature* (2016) 539:390–5. doi:10.1038/nature20132
23. Zhuang ZG, Di GH, Shen ZZ, Ding J, Shao ZM. Enhanced expression of LKB1 in breast cancer cells attenuates angiogenesis, invasion, and metastatic potential. *Mol Cancer Res* (2006) 4:843–9. doi:10.1158/1541-7786.MCR-06-0118
24. Arbiser JL, Petros J, Klafter R, Govindajaran B, Mclaughlin ER, Brown LF, et al. Reactive oxygen generated by Nox1 triggers the angiogenic switch. *Proc Natl Acad Sci U S A* (2002) 99:715–20. doi:10.1073/pnas.022630199
25. Okon IS, Coughlan KA, Zhang C, Moriasi C, Ding Y, Song P, et al. Protein kinase LKB1 promotes RAB7-mediated neuropilin-1 degradation to inhibit angiogenesis. *J Clin Invest* (2014) 124:4590–602. doi:10.1172/JCI75371

26. Miao HQ, Lee P, Lin H, Soker S, Klagsbrun M. Neuropilin-1 expression by tumor cells promotes tumor angiogenesis and progression. *FASEB J* (2000) 14:2532–9. doi:10.1096/fj.00-0250com

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

BRIEF RESEARCH REPORT
published: 13 June 2018
doi: 10.3389/fonc.2018.00199

microRNA-494 Favors HO-1 Expression in Neuroblastoma Cells Exposed to Oxidative Stress in a Bach1-Independent Way

Sabrina Piras[1†], Anna L. Furfaro[1†], Rocco Caggiano[2], Lorenzo Brondolo[1], Silvano Garibaldi[3], Caterina Ivaldo[1], Umberto M. Marinari[1], Maria A. Pronzato[1], Raffaella Faraonio[2,4] and Mariapaola Nitti[1]**

[1] Department of Experimental Medicine, University of Genoa, Genoa, Italy, [2] Department of Molecular Medicine and Medical Biotechnologies, University of Naples "Federico II", Naples, Italy, [3] Department of Internal Medicine, Cardiology, Ospedale Policlinico San Martino, University of Genoa, Genoa, Italy, [4] CEINGE-Biotecnologie Avanzate, Naples, Italy

OPEN ACCESS

Edited by:
Giuseppe Filomeni,
Danish Cancer Society,
Denmark

Reviewed by:
Luisa Rossi,
Università di Roma Tor Vergata, Italy
Sara Baldelli,
Università telematica San Raffaele, Italy
Keith R. Laderoute,
SRI International, United States

***Correspondence:**
Raffaella Faraonio
raffaella.faraonio@unina.it;
Mariapaola Nitti
mariapaola.nitti@unige.it

[†]These authors have contributed equally to this work.

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: 27 February 2018
Accepted: 17 May 2018
Published: 13 June 2018

Citation:
Piras S, Furfaro AL, Caggiano R, Brondolo L, Garibaldi S, Ivaldo C, Marinari UM, Pronzato MA, Faraonio R and Nitti M (2018) microRNA-494 Favors HO-1 Expression in Neuroblastoma Cells Exposed to Oxidative Stress in a Bach1-Independent Way. Front. Oncol. 8:199. doi: 10.3389/fonc.2018.00199

Heme oxygenase 1 (HO-1) is crucially involved in cell adaptation to oxidative stress and has been demonstrated to play an important role in cancer progression and resistance to therapies. We recently highlighted that undifferentiated neuroblastoma (NB) cells are prone to counteract oxidative stress through the induction of HO-1. Conversely, differentiated NB cells were more sensitive to oxidative stress since HO-1 was scarcely upregulated. In this work, we investigated the role played by miR-494, which has been proved to be involved in cancer biology and in the modulation of oxidative stress, in the upregulation of HO-1. We showed that NB differentiation downregulates miR-494 level. In addition, endogenous miR-494 inhibition in undifferentiated cells impairs HO-1 induction in response to exposure to 500 μM H_2O_2, reducing the number of viable cells. The analysis of Bach1 expression did not reveal any significant modifications in any experimental conditions tested, proving that the impairment of HO-1 induction observed in cells treated with miR-494 inhibitor and exposed to H_2O_2 is independent from Bach1. Our results underline the role played by miR-494 in favoring HO-1 induction and cell adaptation to oxidative stress and contribute to the discovery of new potential pharmacological targets to improve anticancer therapies.

Keywords: heme oxygenase 1, neuroblastoma, miR-494, oxidative stress, Bach1

INTRODUCTION

Heme oxygenase 1 (HO-1) is a 32-kDa inducible enzyme belonging to the HO system, which catalyzes the degradation of the iron-containing molecule heme, leading to the generation of free iron (Fe^{2+}), carbon monoxide (CO), and biliverdin. Biliverdin reductase converts biliverdin into bilirubin (1) and ferritin quenches free iron (2). Overall, ferritin, CO, and bilirubin exert strong antioxidant, anti-apoptotic, and anti-inflammatory effects (3). Different activators are involved in HO-1 induction and the nuclear factor erythroid 2-related factor 2 (Nrf2) is considered the most important (4, 5). Moreover, Keap1 by favoring Nrf2 proteasomal degradation, and Bach1 by preventing Nrf2 binding to the promoter region of HO-1, work as HO-1 repressors (6–8).

A sustained HO-1 expression in cancer correlates with a high degree of malignancy (e.g., aggressiveness, metastatic, and angiogenetic potential), although the pro-tumorigenic role of HO-1 seems to be tumor specific and tissue specific (9, 10). In the treatment of highly aggressive neuroblastoma

(NB), the upregulation of HO-1 limits the efficacy of bortezomib (11, 12) suggesting HO-1 inhibition may represent a molecular target in the clinical strategies against NB (13, 14).

By modulating the expression of many different proteins, microRNAs (miRs) supervise and integrate numerous signaling pathways and their involvement has been postulated in various physiological and pathophysiological processes, from differentiation to senescence or oncogenesis (15–17). Strong evidence supports the notion that miRs can behave as oncogenes or tumor suppressor genes (18), and given the important role of oxidative stress response in tumorigenesis, understanding miR regulation in this condition is of major interest. Since a role played by miR-494 in the modulation of oxidative stress has been demonstrated in other contexts (19, 20), but no studies have been conducted in NB cells so far. In this work, we aimed at investigating the functional role of miR-494 in NB cell response to oxidative stress, focusing on its involvement in HO-1 induction.

MATERIALS AND METHODS

Cell Culture and Differentiation

SH-SY5Y and SK-N-BE(2C) NB cells were cultured in RPMI 1640 medium (Euroclone, Italy) supplemented with 10% fetal bovine serum (Euroclone), 2 mM glutamine (Sigma-Aldrich, Italy), 1% amphotericin B (Sigma-Aldrich), and 1% penicillin/streptomycin (Sigma-Aldrich). Cells were differentiated by growth in the same medium supplemented with 10 μM all-trans retinoic acid (ATRA) (Sigma-Aldrich) for 4 days, up to 8 days. Differentiation was monitored by checking morphological changes such as neurite elongation and biochemical markers such as MAP2 and NeuroD1 expression (21, 22).

RNA Extraction and microRNA Level Evaluation

Total RNA was extracted using TRIZOL reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA templates for evaluation of mature miR levels were obtained from input RNAs (10 ng) using TaqMan™ Advanced miR cDNA Synthesis Kit (Thermo Fisher Scientific, USA, Cat. No. A28007) following the manufacturer's protocol. Real-time quantitative PCR for hsa-miR-494, hsa-miR-128, hsa-miR-425-5p, and hsa-let7g-5p was performed in triplicate on diluted cDNA templates (1:10) by using the TaqMan® Advanced miR Assays (Thermo Fisher Scientific, Cat. No. A25576). hsa-miR-425-5p and hsa-let7g-5p were used as endogenous reference miRs. Relative quantification of miR expression levels was performed according to the ΔΔCt method.

Transfection of microRNA Inhibitors

SH-SY5Y cells were transiently transfected with 100 pmol of miR-494 inhibitor (miRCURY LNA miR inhibitor—hsa-miR-494-3p, QIAGEN, Hilder, Germany) and miR inhibitor Control (mirCURY LNA miR inhibitor Control—negative Control A, QIAGEN) by using Lipofectamine 2000 (Life Technologies) following the manufacturer's instruction. 96 h after transfection, cells were treated with 500 μM H_2O_2 for further 6 h to assess Bach1 levels, or for further 24 h to assess HO-1 levels and cell viability.

Cell Viability Assay

Cell viability was evaluated by Trypan blue assay as previously described (12).

Reactive Oxygen Species (ROS) Evaluation

Evaluation of ROS was performed by using 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich) assay. After treatments, cells stained with 5 μM DCFH-DA for 30 min at 37°C were analyzed by FACS (Attune™ Acoustic Focusing Flow Cytometer, Thermo Fisher Scientific). Values are expressed as arbitrary units of fluorescence.

Immunoblotting

Total protein lysates, prepared by using RIPA buffer (13), were subjected to electrophoresis on SDS-polyacrylamide gel (Mini protean precast TGX gel, Bio-Rad, Milan, Italy) (22). Immunodetection was performed using mouse anti-hnRNPQ (1:1,000, Santa Cruz), rabbit anti-PTEN (1:1,000, Cell Signaling Technology, MA, USA), rabbit anti-Bach1 (1:4,000, Bethyl Lab, Montgomery, TX, USA), and rabbit anti-HO-1 (1:2,000, Origene, Herford, Germany) and specific secondary antibodies (GE Healthcare). The membranes were re-probed with the loading control antibodies, rabbit anti-GAPDH (1:1,000, Santa Cruz) or mouse anti-tubulin (1:2,000, AbCam). The bands were detected by means of an enhanced chemiluminescence system (GE Healthcare) and developed films analyzed using a specific software (GelDoc, Bio-Rad).

Statistical Analyses

Statistical analysis of the differences among mean values ± SEM from three or more experiments was performed by using *t*-test to compare two groups or one-way ANOVA followed by Dunnett's post-test to compare more groups.

RESULTS

ATRA-Induced NB Cell Differentiation Is Associated With Reduced Levels of miR-494

The analysis of miR-494 expression, performed after 4-day exposure to 10 μM ATRA, showed a significant reduction in both SH-SY5Y and SK-N-BE(2C) cell lines (**Figure 1A**). SH-SY5Y cells increase the expression of differentiation markers already after 4-day exposure to ATRA, as widely proved (21–23). However, since SK-N-BE(2C) need more time to complete ATRA-induced differentiation (24), miR evaluation was also performed after 6- and 8-day exposure to 10 μM ATRA on SK-N-BE(2C) cells. In these conditions, only a small further decrease of miR-494 expression has been observed (Figure S1a in Supplementary

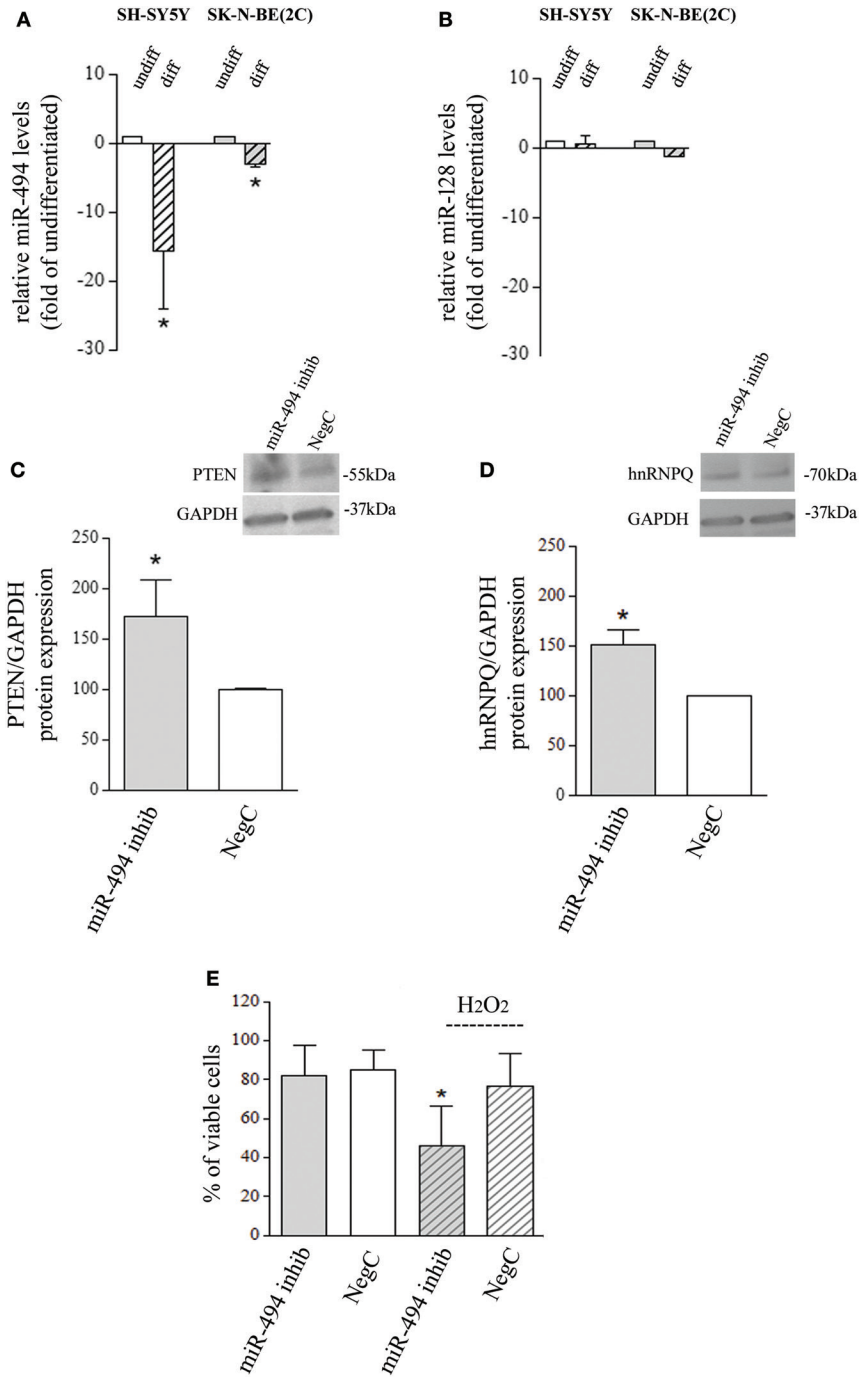


FIGURE 1 | miR-494 downregulation occurs in neuroblastoma (NB) differentiation and modify cell response to H_2O_2. **(A)** Expression levels of mature miR-494 in undifferentiated or all-trans retinoic acid (ATRA)-differentiated SH-SY5Y and SK-N-BE(2C) NB cells. hsa-miR-425-5p and hsa-let7g-5p were used as endogenous reference miRs. Results are reported as relative to the values obtained in untreated control cells, which was set equal to 1. Statistical analysis: $n = 3$; $^*p < 0.05$ vs undifferentiated. **(B)** Expression levels of mature miR-128 in undifferentiated or ATRA-differentiated SH-SY5Y and SK-N-BE(2C) NB cells. hsa-miR-425-5p and hsa-let7g-5p were used as endogenous reference miRs. Results are reported as relative to the values obtained in untreated control cells, which was set equal to 1. Statistical analysis: $n = 3$. No significant differences. **(C)** WB analysis of PTEN. GAPDH expression has been used as loading control. 40 µg of protein has been loaded. The bands show the most representative experiment. Statistical analysis: $n = 3$; $^*p < 0.05$ vs NegC. **(D)** WB analysis of hnRNPQ. GAPDH expression has been used as loading control. 40 µg of protein has been loaded. The bands show the most representative experiment. Statistical analysis: $n = 2$; $^*p < 0.05$ vs NegC. **(E)** Percentage of viable cells (Trypan blue analysis) after miR-494 inhibition and 24 h exposure to 500 µM H_2O_2. Statistical analysis: $n = 4$; $^*p < 0.05$ vs NegC and miRNA 494 inhibitor.

Material). The expression of miR-128 has been also analyzed due to its involvement in stress response and differentiation (25, 26), but no changes were observed in both cell lines after differentiation (**Figure 1B**). The following experiments have been carried out on SH-SY5Y NB cells which strongly downregulated miR-494 (−10-folds vs undifferentiated) in the shortest experimental time (4 days).

miR-494 Inhibition Modifies Cell Responses to Oxidative Stress

To evaluate whether the reduction of endogenous miR-494 could modify NB cell sensitivity to oxidative stress, cells were transfected with a specific miR-494 inhibitor and then exposed to 500 μM H_2O_2. The effectiveness of miR-494 inhibition was checked by evaluating the protein levels of two miR-494 targets, namely, PTEN and hnRNPQ that resulted upregulated of about 50% (**Figures 1C,D**). The analysis of viable cells revealed no changes induced by miR-494 inhibition itself, in comparison to cells transfected with a NegC. Conversely, miR-494 inhibition significantly decreased the percentage of viable cells after the exposure to 500 μM H_2O_2 (**Figure 1E**). The analysis of markers of apoptosis such as BAX and PARP did not show any changes (Figures S1b and S1c in Supplementary Material) and this led us to rule out the occurrence of apoptosis. These results indicate that the expression of miR-494 in undifferentiated NB cells favors cell adaptation/response to oxidative stress.

miR-494 Inhibition Impairs HO-1 Induction in Response to Oxidative Stress

To investigate whether a reduced expression of miR-494 influences oxidative stress response, NB cells transfected with miR-494 inhibitor or NegC were treated with H_2O_2 and ROS levels and HO-1 expression were evaluated. ROS levels were increased only in cells treated with miR-494 inhibitor and exposed to H_2O_2 (**Figure 2A**). In this experimental condition, no significant induction of HO-1 has been observed (**Figure 2B**). Conversely, in NB cells transfected with NegC, the exposure to H_2O_2 was able to significantly increase the expression of HO-1, and the level of ROS was not significantly modified.

The ubiquitination pattern was also analyzed but no changes were detected in any experimental conditions (Figure S1d in Supplementary Material).

In silico analyses predicted Bach1 as a target of miR-494 with two putative sites within its 3′UTR (**Figure 2C**). Thus, we checked the protein levels of Bach1. WB analysis showed that miR-494 inhibition did not modify Bach1 expression after H_2O_2 exposure (**Figure 2D**). Different Bach1 post-translational modifications have also been analyzed; ubiquitination and sumoylation were not detected and Bach1 acetylation was not modified in any experimental conditions (Figure S1e in Supplementary Material). These results show no involvement of Bach1 in the miR-494 dependent HO-1 regulation.

Furthermore, Keap1 levels have been also checked but no changes were detected in any experimental conditions (Figure S1f in Supplementary Material), proving no involvement of Nrf2 in this context.

DISCUSSION

In this work, we pointed out the involvement of miR-494 in the upregulation of HO-1 in NB cell response to oxidative stress. We took into consideration two miRs, such as miR-128 and miR-494. Indeed, miR-128 has been demonstrated to be involved in NB differentiation (26) and response to oxidative stress (25) but we did not observe any modification of miR-128 levels in the different experimental conditions we tested. Thus, we evaluated miR-494 which, from bioinformatics analyses, was predicted to have two putative binding sites on Bach1 3′UTR, the main repressor of HO-1 transcription.

In numerous contexts, miR-494 functions as tumor suppressor gene and has been linked to the induction of senescent phenotype in normal cells (19, 27) but in other contexts it correlates with tumor aggressiveness and progression (28). To the best of our knowledge, there has been no evidence of miR-494 expression in NB so far. We demonstrated that miR-494 is expressed in two undifferentiated NB cell lines and undergoes a significant downregulation after ATRA-induced differentiation. The reduction is dramatic for SH-SY5Y cells that easily differentiate in response to ATRA and minor but always significant in SK-N-BE(2C) which shown medium sensitivity to ATRA (24). There is only a paper in literature showing that the expression of miR-494 is upregulated by ATRA in the acute myeloid leukemia cell line HL-60 (29), and this lets us hypothesize that there may be a cell-type-specific regulation for miR-494. Thus, we further analyzed SH-SY5Y cells which, from our previous works, have been proved to increase their sensitivity to oxidative stress after differentiation (22, 30), investigating a possible correlation with the miR-494 downregulation. We observed that miR-494 inhibition in undifferentiated cells significantly reduced the number of viable cells after exposure to H_2O_2. The role of miR-494 in cell survival is controversial, depending on the cellular context where miR operates and on the accessibility of its targets. As also shown in our work, miR-494 inhibition is able to increase PTEN expression and, potentially, to antagonize the AKT survival pathway, as proved in other contexts (31, 32). However, the modulation of miR-494-PTEN signaling under stress condition has not yet been investigated.

Next, we provided evidence that endogenous miR-494 inhibition impairs HO-1 upregulation in response to oxidative stress, similar to what we have already shown in differentiated cells exposed to H_2O_2 (22). In addition, we showed that the lack of HO-1 upregulation correlates with higher ROS levels, highlighting the importance of HO-1 induction in quenching ROS. However, the analysis of Bach1 expression revealed that there are no significant modifications in the level of Bach1 in response to H_2O_2 in NB cells treated with miR-494 inhibitor compared with cells transfected with NegC. Moreover, there are no changes in Bach1 ubiquitination, sumoylation, and acetylation in any experimental conditions examined. Thus, miR-494 could contribute through a Bach1-independent mechanism to modulate HO-1 expression under stress response. It has been already demonstrated that HO-1 transcription can be controlled *via* Bach1 turnover in the absence or presence of oxidative stress and can also be

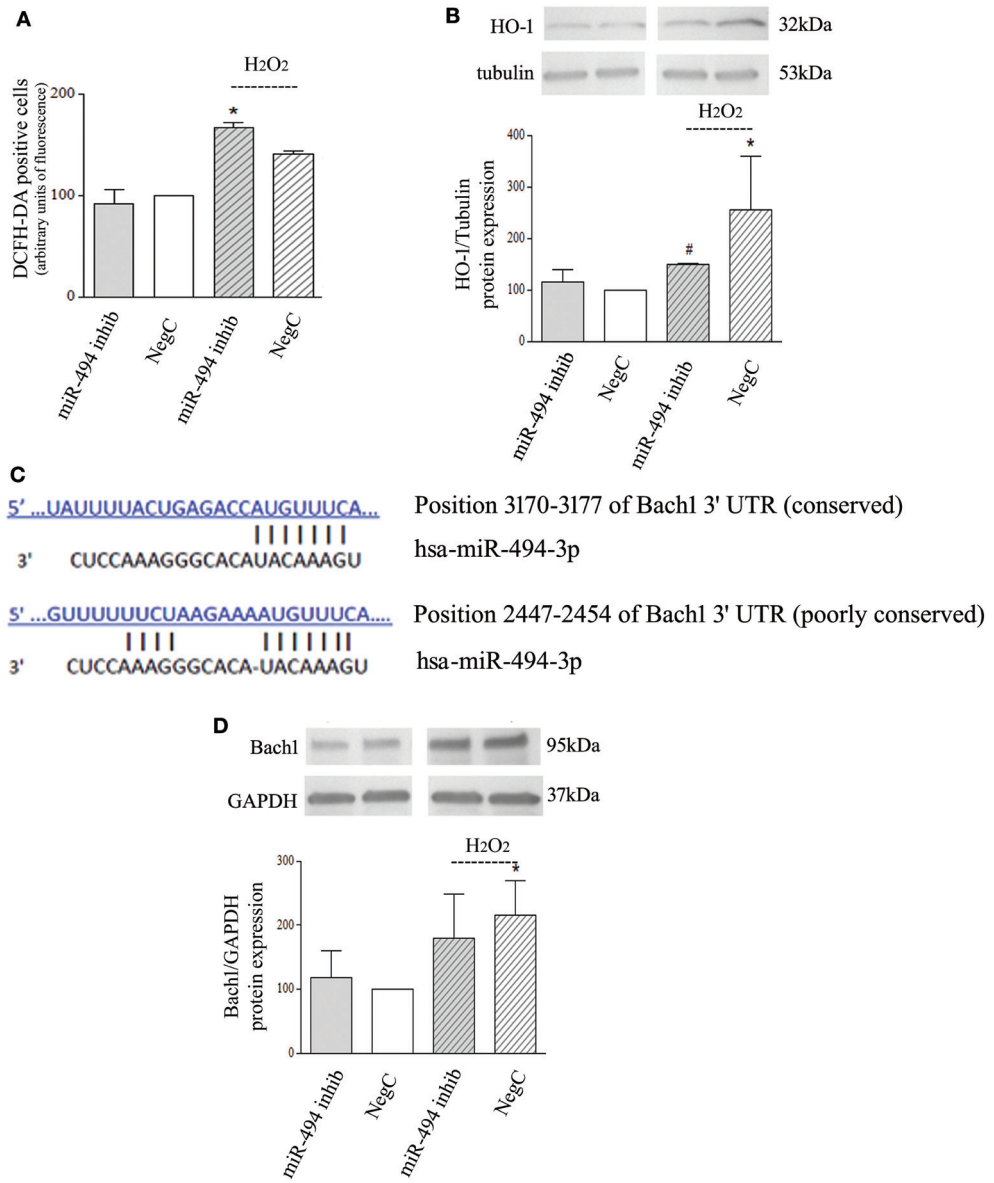


FIGURE 2 | miR-494 inhibition impairs heme oxygenase 1 (HO-1) induction in response to H_2O_2 in Bach1-independent way. **(A)** Positivity to DCFH-DA has been measured by cytofluorimetric analyses after miR-494 inhibition and 6 h exposure to 500 μM H_2O_2. Statistical analysis: $n = 2$; $^*p < 0.05$ vs NegC. **(B)** WB analysis of HO-1 expression. Tubulin expression has been used as loading control. 30 μg of protein has been loaded. The bands show the most representative experiment. Statistical analysis: $n = 3$; $^*p < 0.05$ vs NegC. $^\#p < 0.05$ vs NegC + H_2O_2. **(C)** The human Bach1 3′UTR contains two seed sites for miR-494. The sequence alignments were predicted using TargetScan. **(D)** WB analysis of Bach1 expression. GAPDH expression has been used as loading control. 30 μg of protein has been loaded. The bands show the most representative experiment. Statistical analysis: $n = 4$; $^*p < 0.05$ vs NegC.

insensitive to Bach1-mediated repression (33). Moreover, AKT-dependent HO-1 induction has been already proved (34), and miR-494-PTEN might crucially modulate it. To validate this hypothesis, further analyses are needed.

AUTHOR CONTRIBUTIONS

SP, ALF, SG, RF, and MN conceived and designed the experiments. SP, ALF, RC, LB, SG, and CI conducted the experiments. SP, ALF, SG, RF, and MN analyzed the results. UMM, MAP, RF, and MN contributed reagents/materials/analysis. SP, ALF, UMM, RF, and MN wrote the paper. All the authors reviewed the manuscript.

FUNDING

This study was supported by grants from University of Genoa (to MN).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fonc.2018.00199/full#supplementary-material.

FIGURE S1 | **(A)** Expression levels of mature miR-494 and miR-128 in undifferentiated and 6- or 8-day-differentiated SK-N-BE(2C) NB cells. hsa-miR-425-5p and hsa-let7g-5p have been used as endogenous reference miRs. Results are reported as relative to the values obtained in untreated undifferentiated cells which was set equal to 1. Statistical analysis: $n = 3$; $^*p < 0.05$ vs undifferentiated. **(B)** WB analysis of BAX in SH-SY5Y cells treated with miR-494 inhibitor and exposed to 500mM H_2O_2, as indicated. GAPDH expression has been used as loading control. 10 mg of proteins was loaded. The blots show one representative experiment. Statistical analysis: $n = 3$; no significant differences. **(C)** WB analysis of PARP in SH-SY5Y cells treated with miR-494 inhibitor and exposed to 500mM H_2O_2, as indicated. GAPDH expression has been used as loading control. 50 mg of proteins was loaded. The blots show one representative experiment. Statistical analysis: $n = 2$; no significant differences. **(D)** WB analysis of ubiquitination in SH-SY5Y cells treated with miR-494 inhibitor and exposed to 500mM H_2O_2, as indicated. 20 mg of proteins was loaded. The blot shows one representative experiment. **(E)** Analysis of Bach1 post-translational modifications in SH-SY5Y cells treated with miR-494 inhibitor and exposed to 500mM H_2O_2 for 6 h. 300 mg of protein lysate was immunoprecipitated using anti Bach1 and loaded in electrophoresis (ip). An aliquot of supernatant collected after the first step of immunoprecipitation was loaded in electrophoresis (sn). WB detection was performed as indicated. The blots show the most representative experiment. **(F)** WB analysis of Keap1 in SH-SY5Y cells treated with miR-494 inhibitor and exposed to 500mM H_2O_2 as indicated. GAPDH expression has been used as loading control. 40 mg of proteins was loaded. The blots show one representative experiment. Statistical analysis: $n = 3$; no significant differences.

REFERENCES

1. Maines MD. Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. *FASEB J* (1988) 2:2557–68. doi:10.1096/fasebj.2.10.3290025
2. Cheng HT, Yen CJ, Chang CC, Huang KT, Chen KH, Zhang RY, et al. Ferritin heavy chain mediates the protective effect of heme oxygenase-1 against oxidative stress. *Biochim Biophys Acta* (2015) 1850:2506–17. doi:10.1016/j.bbagen.2015.09.018
3. Loboda A, Damulewicz M, Pyza E, Jozkowicz A, Dulak J. Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism. *Cell Mol Life Sci* (2016) 73:3221–47. doi:10.1007/s00018-016-2223-0
4. Hayes JD, Dinkova-Kostova AT. The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. *Trends Biochem Sci* (2014) 39:199–218. doi:10.1016/j.tibs.2014.02.002
5. Nguyen T, Nioi P, Pickett CB. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *J Biol Chem* (2009) 284:13291–5. doi:10.1074/jbc.R900010200
6. Itoh K, Mimura J, Yamamoto M. Discovery of the negative regulator of Nrf2, Keap1: a historical overview. *Antioxid Redox Signal* (2010) 13:1665–78. doi:10.1089/ars.2010.3222
7. Kobayashi M, Yamamoto M. Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. *Antioxid Redox Signal* (2005) 7:385–94. doi:10.1089/ars.2005.7.385
8. Villeneuve NF, Lau A, Zhang DD. Regulation of the Nrf2-Keap1 antioxidant response by the ubiquitin proteasome system: an insight into cullin-ring ubiquitin ligases. *Antioxid Redox Signal* (2010) 13:1699–712. doi:10.1089/ars.2010.3211
9. Nitti M, Piras S, Marinari UM, Moretta L, Pronzato MA, Furfaro AL. HO-1 induction in cancer progression: a matter of cell adaptation. *Antioxidants (Basel)* (2017) 6:E29. doi:10.3390/antiox6020029
10. Was H, Dulak J, Jozkowicz A. Heme oxygenase-1 in tumor biology and therapy. *Curr Drug Targets* (2010) 11:1551–70. doi:10.2174/1389450111009011551
11. Furfaro AL, Piras S, Passalacqua M, Domenicotti C, Parodi A, Fenoglio D, et al. HO-1 up-regulation: a key point in high-risk neuroblastoma resistance to bortezomib. *Biochim Biophys Acta* (2014) 1842:613–22. doi:10.1016/j.bbadis.2013.12.008
12. Furfaro AL, Piras S, Domenicotti C, Fenoglio D, De Luigi A, Salmona M, et al. Role of Nrf2, HO-1 and GSH in neuroblastoma cell resistance to bortezomib. *PLoS One* (2016) 11:e0152465. doi:10.1371/journal.pone.0152465
13. Furfaro AL, Macay JR, Marengo B, Nitti M, Parodi A, Fenoglio D, et al. Resistance of neuroblastoma GI-ME-N cell line to glutathione depletion involves Nrf2 and heme oxygenase-1. *Free Radic Biol Med* (2012) 52:488–96. doi:10.1016/j.freeradbiomed.2011.11.007
14. Furfaro AL, Traverso N, Domenicotti C, Piras S, Moretta L, Marinari UM, et al. The Nrf2/HO-1 axis in cancer cell growth and chemoresistance. *Oxid Med Cell Longev* (2016) 2016:1958174. doi:10.1155/2016/1958174
15. Paul P, Chakraborty A, Sarkar D, Langthasa M, Rahman M, Bari M, et al. Interplay between miRNAs and human diseases. *J Cell Physiol* (2018) 233:2007–18. doi:10.1002/jcp.25854
16. Tüfekci KU, Oner MG, Meuwissen RLJ, Genç S. The role of microRNAs in human diseases. *Methods Mol Biol* (2014) 1107:33–50. doi:10.1007/978-1-62703-748-8_3
17. Tüfekci KU, Meuwissen RLJ, Genç S. The role of microRNAs in biological processes. *Methods Mol Biol* (2014) 1107:15–31. doi:10.1007/978-1-62703-748-8_2
18. Peng Y, Croce CM. The role of microRNAs in human cancer. *Signal Transduct Target Ther* (2016) 1:15004. doi:10.1038/sigtrans.2015.4
19. Faraonio R, Salerno P, Passaro F, Sedia C, Iaccio A, Bellelli R, et al. A set of miRNAs participates in the cellular senescence program in human diploid fibroblasts. *Cell Death Differ* (2012) 19:713–21. doi:10.1038/cdd.2011.143
20. Xiong R, Wang Z, Zhao Z, Li H, Chen W, Zhang B, et al. MicroRNA-494 reduces DJ-1 expression and exacerbates neurodegeneration. *Neurobiol Aging* (2014) 35:705–14. doi:10.1016/j.neurobiolaging.2013.09.027
21. Nitti M, Furfaro AL, Cevasco C, Traverso N, Marinari UM, Pronzato MA, et al. PKC delta and NADPH oxidase in retinoic acid-induced neuroblastoma cell differentiation. *Cell Signal* (2010) 22:828–35. doi:10.1016/j.cellsig.2010.01.007
22. Piras S, Furfaro AL, Brondolo L, Passalacqua M, Marinari UM, Pronzato MA, et al. Differentiation impairs Bach1 dependent HO-1 activation and increases sensitivity to oxidative stress in SH-SY5Y neuroblastoma cells. *Sci Rep* (2017) 7:7568. doi:10.1038/s41598-017-08095-7
23. Piras S, Furfaro AL, Piccini A, Passalacqua M, Borghi R, Carminati E, et al. Monomeric Abeta1-42 and RAGE: key players in neuronal differentiation. *Neurobiol Aging* (2014) 35:1301–8. doi:10.1016/j.neurobiolaging.2014.01.002
24. Yao P-L, Chen L, Dobrzański TP, Zhu B, Kang B-H, Müller R, et al. Peroxisome proliferator-activated receptor-β/δ inhibits human neuroblastoma cell tumorigenesis by inducing p53- and SOX2-mediated cell differentiation. *Mol Carcinog* (2017) 56:1472–83. doi:10.1002/mc.22607
25. Caggiano R, Cattaneo F, Moltedo O, Esposito G, Perrino C, Trimarco B, et al. miR-128 is implicated in stress responses by targeting MAFG in skeletal muscle cells. *Oxid Med Cell Longev* (2017) 2017:9308310. doi:10.1155/2017/9308310
26. Evangelisti C, Florian MC, Massimi I, Dominici C, Giannini G, Galardi S, et al. miR-128 up-regulation inhibits reelin and DCX expression and reduces neuroblastoma cell motility and invasiveness. *FASEB J* (2009) 23:4276–87. doi:10.1096/fj.09-134965
27. Comegna M, Succoio M, Napolitano M, Vitale M, D'Ambrosio C, Scaloni A, et al. Identification of miR-494 direct targets involved in senescence of human diploid fibroblasts. *FASEB J* (2014) 28:3720–33. doi:10.1096/fj.13-239129
28. Zhang Y, Guo L, Li Y, Feng G-H, Teng F, Li W, et al. MicroRNA-494 promotes cancer progression and targets adenomatous polyposis coli in colorectal cancer. *Mol Cancer* (2018) 17:1. doi:10.1186/s12943-017-0753-1
29. Jian P, Li ZW, Fang TY, Jian W, Zhuan Z, Mei LX, et al. Retinoic acid induces HL-60 cell differentiation via the upregulation of miR-663. *J Hematol Oncol* (2011) 4:20. doi:10.1186/1756-8722-4-20

30. Nitti M, Furfaro AL, Traverso N, Odetti P, Storace D, Cottalasso D, et al. PKC delta and NADPH oxidase in AGE-induced neuronal death. *Neurosci Lett* (2007) 416:261–5. doi:10.1016/j.neulet.2007.02.013
31. Li X-T, Wang H-Z, Wu Z-W, Yang T-Q, Zhao Z-H, Chen G-L, et al. miR-494-3p regulates cellular proliferation, invasion, migration, and apoptosis by PTEN/AKT signaling in human glioblastoma cells. *Cell Mol Neurobiol* (2015) 35:679–87. doi:10.1007/s10571-015-0163-0
32. Wang Y, Xu J, Gao G, Li J, Huang H, Jin H, et al. Tumor suppressor NFκB2 p100 interacts with ERK2 and stabilizes PTEN mRNA via inhibition of miR-494. *Oncogene* (2016) 35:4080–90. doi:10.1038/onc.2015.470
33. Tan M-KM, Lim H-J, Bennett EJ, Shi Y, Harper JW. Parallel SCF adaptor capture proteomics reveals a role for SCFFBXL17 in NRF2 activation via BACH1 repressor turnover. *Mol Cell* (2013) 52:9–24. doi:10.1016/j.molcel.2013.08.018
34. Ku H-C, Lee S-Y, Yang K-C, Kuo Y-H, Su M-J. Modification of caffeic acid with pyrrolidine enhances antioxidant ability by activating AKT/HO-1 pathway in heart. *PLoS One* (2016) 11:e0148545. doi:10.1371/journal.pone.0148545

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 23 April 2018
doi: 10.3389/fonc.2018.00122

Novel Mitochondria-Targeted Furocoumarin Derivatives as Possible Anti-Cancer Agents

Andrea Mattarei[1], Matteo Romio[1], Antonella Managò[2], Mario Zoratti[3,4], Cristina Paradisi[1], Ildikò Szabò[2], Luigi Leanza[2] and Lucia Biasutto[3,4]**

[1] Department of Chemical Sciences, University of Padova, Padova, Italy, [2] Department of Biology, University of Padova, Padova, Italy, [3] CNR Neuroscience Institute, Padova, Italy, [4] Department of Biomedical Sciences, University of Padova, Padova, Italy

Targeting small molecules to appropriate subcellular compartments is a way to increase their selectivity and effectiveness while minimizing side effects. This can be accomplished either by stably incorporating specific "homing" properties into the structure of the active principle, or by attaching to it a targeting moiety *via* a labile linker, i.e., by producing a "targeting pro-drug." Mitochondria are a recognized therapeutic target in oncology, and blocking the population of the potassium channel Kv1.3 residing in the inner mitochondrial membrane (mtKv1.3) has been shown to cause apoptosis of cancerous cells expressing it. These concepts have led us to devise novel, mitochondria-targeted, membrane-permeant drug candidates containing the furocoumarin (psoralenic) ring system and the triphenylphosphonium (TPP) lipophilic cation. The strategy has proven effective in various cancer models, including pancreatic ductal adenocarcinoma, melanoma, and glioblastoma, stimulating us to devise further novel molecules to extend and diversify the range of available drugs of this type. New compounds were synthesized and tested *in vitro*; one of them—a prodrug in which the coumarinic moiety and the TPP group are linked by a bridge comprising a labile carbonate bond system—proved quite effective in *in vitro* cytotoxicity assays. Selective death induction is attributed to inhibition of mtKv1.3. This results in oxidative stress, which is fatal for the already-stressed malignant cells. This compound may thus be a candidate drug for the mtKv1.3-targeting therapeutic approach.

Keywords: Kv1.3, mitochondria-targeting, cancer, triphenylphosphonium, psoralens, pancreatic duct adenocarcinoma

OPEN ACCESS

Edited by:
Giuseppe Filomeni, Danish Cancer Society, Denmark

Reviewed by:
Jiri Neuzil, Griffith University, Australia
Vincenzo Ciminale, Università degli Studi di Padova, Italy

****Correspondence:***
Luigi Leanza
luigi.leanza@unipd.it;
Lucia Biasutto
lucia.biasutto@cnr.it

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *01 February 2018*
Accepted: *04 April 2018*
Published: *23 April 2018*

Citation:
Mattarei A, Romio M, Managò A, Zoratti M, Paradisi C, Szabò I, Leanza L and Biasutto L (2018) Novel Mitochondria-Targeted Furocoumarin Derivatives as Possible Anti-Cancer Agents. Front. Oncol. 8:122. doi: 10.3389/fonc.2018.00122

INTRODUCTION

In therapeutic oncology, the ultimate goal is to cause the death of all cancerous and cancer stem cells while inflicting negligible damage to healthy cells and organs. Several strategies have been adopted in an effort to achieve this difficult result. One is to selectively hit only the unwanted cells with an effective death-inducing treatment. Examples of this approach include focused radiation therapy, the many attempts at delivering drugs selectively to tumoral cells, and immunotherapy. Another is aiming a drug at a molecular target which is expressed specifically by cancer cells and/or whose function is more cogently needed by cancer cells than by healthy ones, including of course malfunctioning oncogenes. An example may be the development of small molecule inhibitors of deregulated oncogenic kinases. A third one may be to exploit an intrinsic characteristic of cancer cells, such as increased aerobic glycolysis (the Warburg effect) or their rapid growth and the associated redox stress

they experience (1). This latter feature is of particular interest for this paper. An elevated production of ROS is a characteristic of rapidly proliferating cells (2). In cancerous cells, it is induced by various mechanisms (1). For example, K-ras hyperactivity has been reported to lead to suppression of respiratory chain (RC) complex-I, ROS generation, mitochondrial dysfunction, and switch to glycolytic metabolism (3, 4). Tumor suppressor p53 on the other hand acts *via* Bcl-2 family proteins to reduce mitochondrial ROS, and its loss or malfunction may thus lead to an increase in their production (5, 6). In fact, anti-apoptotic proteins of the Bcl-2 family, such as Bcl-XL and Bcl-w, reportedly increase mt ROS production. They do this by binding and neutralizing Bax, which reduces ROS by interacting with complex I (7, 8).

This stressed state can be exploited to induce cell death (1, 9–11). Excessive ROS can cause cell death by processes such as apoptosis (12) [mediated, for example, by redox-sensitive apoptosis signal-regulating kinase family members (13)], necrosis (14), and ferroptosis (15). An oxidative stress exceeding the "death threshold" can be achieved either by weakening the cellular antioxidant defenses which keep it within "safe" limits (16)—for example, by inhibiting a member of the peroxiredoxin system (17, 18)—or by increasing it. In turn, this latter option can be achieved either by using drugs which are themselves redox-active [e.g., Q-7BTPI (19)], or by stimulating the cells' ROS-producing apparatuses, such as the mitochondrial RC [e.g., Ref. (20)].

Given their key role in cancer metabolism, progression, and survival (9, 21–26) and in apoptosis, mitochondria are a focus of anti-cancer chemotherapy (27–29). Of relevance here, mitochondrial ion channels are potential targets of strategies aiming to stress cancer cells to death. They influence mitochondrial membrane potential $\Delta\Psi$, ROS production, volume, and ion homeostasis (30). Pharmacological manipulation of mitochondrial ion channels can lead to cell death bypassing the upstream players of intrinsic apoptosis (p53 status, Bax/Bak/Bcl-2 expression and alterations of cytosolic signaling pathways) (31).

In particular, our group has uncovered (32, 33) a crucial role of mitochondrial potassium-selective channel mtKv1.3 blockage by pro-apoptotic Bax in the apoptotic death of cells expressing mtKv1.3, which include many cancer cell lines (34). The other finding this line of research descends from is the observation (35) that 5-(4-phenylbutoxy)psoralen (Psora-4) (**Figure 1**), a membrane-permeant molecule, blocked Kv1.3 with an EC_{50} of 3 nM. A derivative, PAP-1, was less effective but more selective for Kv1.3 vs Kv1.5, which is also often expressed in the mitochondria of cancer cells. We used these compounds to show that pharmacological inhibition of mtKv1.3 could cause the same outcome as inhibition by Bax, i.e., death by apoptosis (36, 37). This outcome is currently understood to result from the following chain of events: stopping the depolarizing K^+ influx causes inner mitochondrial membrane (IMM) hyperpolarization, with ensuing increased ROS level, activation of the mitochondrial permeability transition pore, mitochondrial swelling, loss of transmembrane potential, loss of cytochrome c, and further ROS release (36).

5-(4-phenylbutoxy)psoralen and PAP-1, however, had only a modest effect on cancerous cells when used at pharmacologically meaningful concentrations. To improve their effectiveness we sought to target the drugs to the IMM and mitochondrial matrix. The most effective and popular strategy for mitochondrial targeting relies on conjugating the drug to a lipophilic, membrane-permeant cation, most often triphenylphosphonium (TPP) (38–41). Various drugs based on this design and producing cytotoxic oxidative stress in cancerous cells have already been produced: mitochondria-targeted vitamin E succinate (MitoVES) (42–44), a construct interacting with RC complex-II; MitoMets (45, 46), metformin derivatives inhibiting RC complex I and inducing ROS production; MitoTam (47), based on tamoxifen and likewise acting *via* RC complex-I; mitochondriotropic derivatives of the polyphenols resveratrol (20, 48) and quercetin (19, 49), also causing deadly redox stress in cultured cells *via* the RC or concentration-enhanced autoxidation, respectively. We thus synthesized two TPP-comprising PAP-1 derivatives, PAPTP and PCARBTP, both of which turned out to be promising chemotherapeutic agents, selectively eliminating cancerous cells *in vitro* and in *in vivo* oncological models, including orthotopic melanoma and pancreatic ductal adenocarcinoma (PDAC) (50). These compounds caused the death of pathological cells independently of the expression levels of key pro- or anti-apoptosis proteins, such as Bax, Bak, Bcl-2, or p53. Importantly, they had no significant impact on healthy tissues and cells, including the immune system of mice and humans (50). The structure of PCARBTP combines two concepts: mitochondrial targeting, conferred by TPP, and prodrug function, provided by the carbamate link connecting the two parts of the molecule, PAP-1 and TPP. Since the carbamate group is hydrolyzed over several minutes in a physiological environment, this device allows the delivery of the essentially unmodified active agent to mitochondria.

Given the promising results, we have extended the search for anti-tumoral agents combining the Kv1.3-inhibiting furocoumarin structure and the mitochondriotropic TPP group.

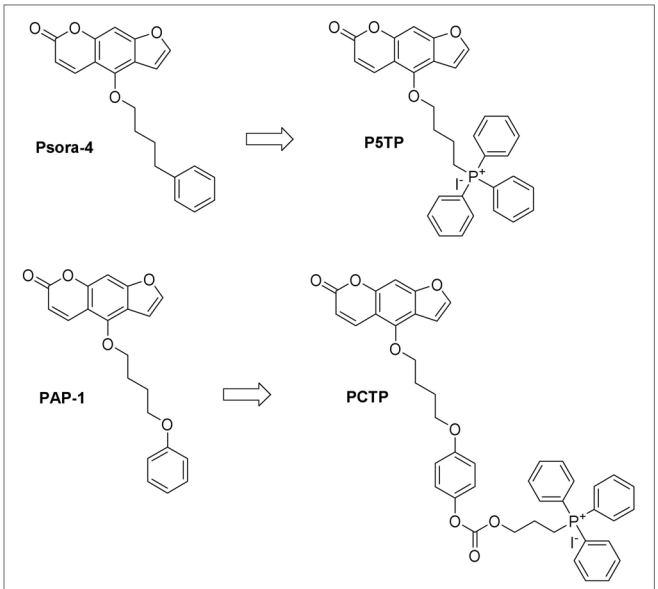


FIGURE 1 | Chemical structures of the compounds studied in this work and their precursors.

MATERIALS AND METHODS

Chemistry

Details of the synthetic procedures used in this study are provided in the Supplementary Material.

Cell Lines

B16F10 cells (ATCC) were grown in Minimum Essential Media (MEM, Thermo Fisher Scientific) supplemented with 10 mM HEPES buffer (pH 7.4), 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin G, 0.1 mg/mL streptomycin, and 1% non-essential amino acids (100× solution; Thermo Fisher Scientific). Lymphocytes (Jurkat, CTLL-2, and K562) were grown in RPMI 1640 (Thermo Fisher Scientific), supplemented as MEM. Medium for CTLL-2 was further supplemented with four units/mL/day of mouse interleukin-2 (IL-2). A panel of pancreatic cancer cell lines (51) was used: BxPC3, AsPC1, Capan-1, and PANC-1. BxPC3 derived from the body of the pancreas of a patient with adenocarcinoma. These cells are not prone to give metastasis and they are poorly differentiated. The other three lines were originally obtained from metastases (AsPC-1, Capan-1) or have considerable metastatic potential (PANC-1). All were provided by ATCC. AsPC1 and BxPC3 were cultured in RPMI-1640 supplemented with 10% FBS "GOLD" (PAA Laboratories/GE Healthcare Life Sciences), 1 mM GlutaMAX, and 1 mM sodium pyruvate (Thermo Fisher Scientific). PANC-1 were cultured in DMEM (4.5 g/L D-glucose) supplemented with 10% FBS "GOLD", 1 mM GlutaMAX and 1 mM sodium pyruvate. Capan-1 cells were grown in IMEM supplemented with 20% FBS "GOLD," 1 mM GlutaMAX, and 1 mM sodium pyruvate. The HPV16-E6E7—immortalized human pancreatic duct epithelial cells (HPDE), kindly provided by Dr. Ming-Sound Tsao (Ontario Cancer Institute, Toronto, ON, Canada) (52) were used as a model for benign pancreatic ductal epithelium. The complete HPDE growth medium was a mixture of 50% RPMI 1640, supplemented with 10% FCS and 1 mM GlutaMAX, and 50% keratinocyte medium SFM (Thermo Fisher Scientific) supplemented with 0.025% bovine pituitary extract, 2.5 mg/L epidermal growth factor (Thermo Fisher Scientific).

Cell Viability and Cell Death Assays

For cell growth/viability MTT assays we used a protocol previously described (36, 50). Briefly, cells were seeded (5–10 × 10^3 cells/well) in standard 96-well plates and allowed to grow in medium (200 µL) for 24 h to ensure attachment. The growth medium was then replaced in the dark with a medium that contained the desired compound (from a stock solution in DMSO) at the final concentration. The final concentration of DMSO was 0.1% or lower in all cases (including controls). To inhibit multiple drug resistance (MDR) "pumps," where indicated we used non-toxic concentrations of cyclosporine H (CSH) (1 µM; Sequoia) and Probenecid (100 µM; Sigma Aldrich) in the case of CTLL-2 cells, and of CSH only (4 µM) for the other cell lines. After incubation for 24 h, CellTiter 96 AQUEOUS One solution (Promega, Italy) was added to each well as indicated by the supplier. Absorbance was measured at 490 nm to detect formazan formation using a Packard Spectra Count 96-well plate reader.

For cell death assays of non-adherent cells, cells were incubated with the test substances for 24 h, washed in HBSS, and resuspended in DMEM without serum and Phenol Red and incubated for 30 min at 37°C in the dark with Annexin-V FLUOS (Roche) (1 µL/200 µL sample). DMSO concentration was <0.1% in all cases. Flow cytometry analysis was carried out after the labeling period with a Becton Dickinson FACS Canto II flow cytometer and data were processed by quadrant statistics using BD VISTA software.

Downregulation of Kv1.3 Expression by siRNA

The sequences for the siRNA targeting human Kv1.3 were coupled to Alexa Fluo 555 (Hs_KCNA3_1 Flexi tube siRNA for Kv1.3 and All-star negative control siRNA as scramble/control; Qiagen). Jurkat cells were transfected by electroporation, as previously reported (36). After 48 h from transfection, cells were treated for 24 h with the various compounds as indicated. Cell death was then evaluated by the binding of fluorescein isothiocyanate (FITC)-labeled Annexin-V and FACS analysis.

Mitochondrial Morphology, Membrane Potential, and ROS Production

Mitochondrial morphology was studied in melanoma B16F10 cells. 1 × 10^5 cells were seeded in a 6-well plate with 2 mL of complete medium. After 24 h medium was replaced with 1 mL HBSS supplemented with 500 nM Mitotracker green (Thermo Scientific). Cells were incubated at 37°C in the dark for 20 min and then the mitochondrial network was observed by confocal microscopy using a Leica DMI6000 fluorescence microscope with confocal settings (Leica Microsystem, Wetzalar, Germany).

Mitochondrial membrane potential and ROS production were measured in leukemic Jurkat T cells. 5 × 10^5 cells were resuspended in 300 µL of HBSS supplemented either with 20 nM TMRM or 1 µM MitoSOX. Cells were incubated for 20 min at 37°C in the dark. Then, cells were diluted by the addition of further 1.2 mL of HBSS and analyzed by FACS (FACSanto II, Beckton Dickson).

Western Blot

Kv1.3 protein expression was assessed after transfection with control ("scramble") and anti-Kv1.3 siRNA. Jurkat cells from parallel siRNA experiments were lysed overnight refrigeration at −80°C in lysis buffer (25 mM Tris pH 7.8 + 2.5 mM EDTA + 10% glycerol + 1% NP-40 + 2 mM DTT). After thawing, debris was centrifuged off at 20,000 × *g* for 10 min at 4°C. Supernatants were collected and protein concentration was determined using the BCA method in a 96-well plate (200 µL total volume for each well) incubating at 37°C in the dark for 30 min. Absorbance at 540 nm was measured by a Packard Spectra Count 96-well plate reader. Proteins were separated by SDS-PAGE in a 10% polyacrylamide gel. After separation by electrophoresis, gels were blotted overnight at 4°C onto polyvinylidene fluoride membranes and then membranes were blocked with a 10% solution of defatted milk and were incubated with the following primary antibodies overnight at 4°C: anti-Kv1.3 (1:200, rabbit polyclonal, Alomone Labs APC-101); anti-GAPDH (1:1,000, mouse monoclonal,

Millipore MAB374). After washing, the membranes were developed using corresponding anti-mouse or anti-rabbit secondary antibodies (Calbiochem). The antibody signal was detected with enhanced chemiluminescence substrate (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific).

Statistics

Statistical significance of the effects was assessed by paired *t*-test or two-way ANOVA analysis.

RESULTS AND DISCUSSION

One of the concepts we tested was to minimize the changes to the structure of the "parent" drug, Psora-4, while still turning it into a mitochondria-targeted drug. Thus, we simply substituted the distal phenyl ring with the TPP group (P5TP, **Figure 1**).

A second approach was that of attaching a mitochondria-targeting group to PAP-1. This strategy had proved successful when attaching the TPP moiety *via* a stable bond system or *via* a labile linker comprising a carbamate group (50). We thus tested another labile "joint," the carbonate group, producing PCTP (**Figure 1**).

The rationale for the preparation of these new derivatives was based on previous studies which underlined the importance of not altering the planar furocoumarin system. The modification at position five of the psoralen scaffold did not affect the ability of

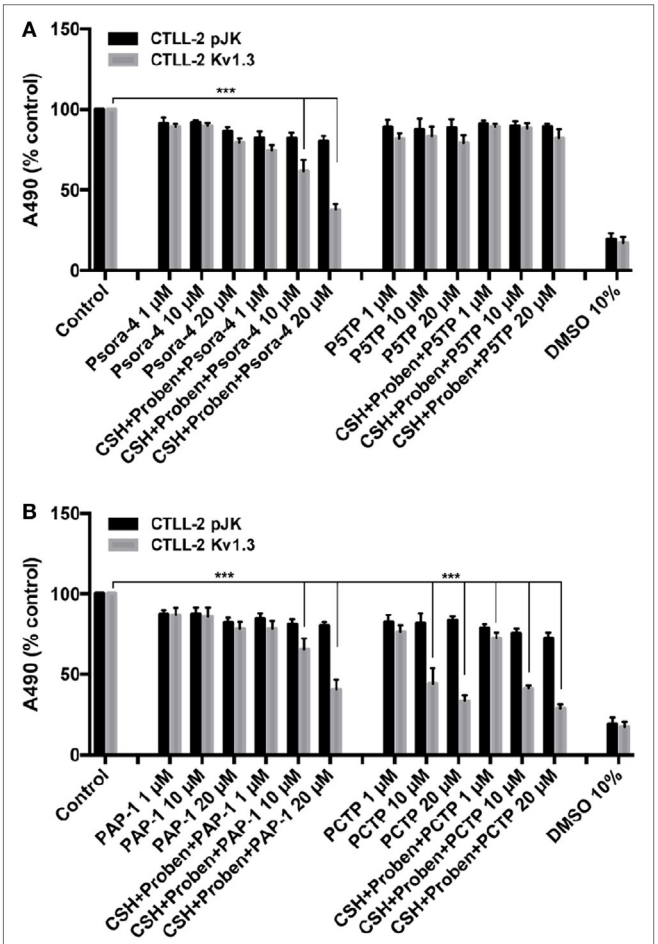


FIGURE 2 | PCTP selectively eliminates Kv1.3-expressing cells. Murine lymphocyte CTLL-2 cells were transfected either with the empty vector (CTLL-2 pJK) or with the expression vector for Kv1.3 (CTLL-2 Kv1.3). Cell viability was assessed by MTT assay after 24 h of incubation with Psora-4, P5TP **(A)**, PAP-1, or PCTP **(B)** at different concentrations either without or with the addition of 1 μM cyclosporine H and 100 μM probenecid as multidrug resistance pumps inhibitors. DMSO 10% was used as positive control, since it is toxic at this concentration. Results are reported as mean percentage of viable cells normalized with respect to untreated cells ($n = 3$; $^{***}p < 0.001$).

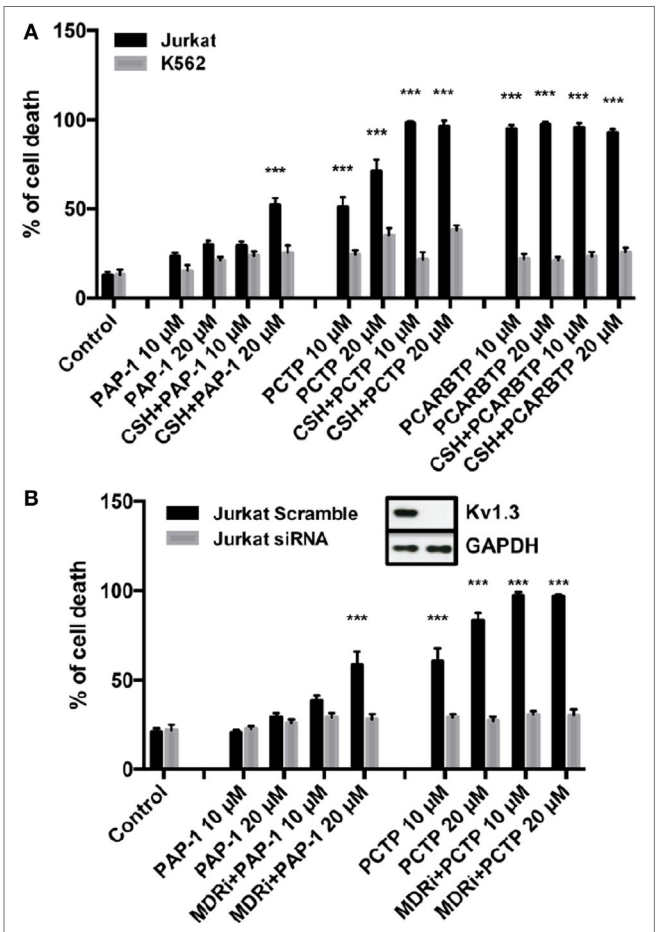


FIGURE 3 | PCTP is specific in inducing cell death by Kv1.3 inhibition. **(A)** Jurkat T lymphocytes and leukemic K562 cells were treated for 24 h with PAP-1, PCTP, or PCARBTP at the indicated concentrations with or without the addition of 4 μM cyclosporine H as multidrug resistance pumps inhibitor. Cell death by apoptosis was then determined by incubation with fluorescein isothiocyanate-labeled annexin-V for 20 min at 37°C in the dark. Annexin-V positive cells were measured by FACS analysis ($n = 3$; $^{***}p < 0.001$ vs control). **(B)** Jurkat cells were either transfected with a control siRNA (Scramble) or siRNA against Kv1.3 (siRNA) and after 48 h from the transfection they were treated as in **(A)** with PAP-1 and PCTP ($n = 3$; $^{***}p < 0.001$ vs control). Insert: Kv1.3 downregulation was assessed by Western blot after siRNA transfection. A representative image is shown of three independent observations. GAPDH was used as loading control.

the molecule to block the potassium cation inside the cavity of the channel as proposed by Zimin and coworkers (53).

For both derivatives the synthesis started with the natural compound bergapten (5-methoxypsoralen) and utilized the key intermediate PSBI (Figure S1 in Supplementary Material). The conversion of bergapten to PSBI was achieved by demethylation to bergaptol (2) promoted by BBr_3, followed by alkylation with 1-bromo-4-chlorobutane to obtain the chloro-intermediate 3 and substitution of chloride with iodide *via* the Finkelstein reaction. Details are provided in Supplementary Material.

PCTP underwent a slow hydrolysis in DMEM, at pH 7.4 and 37°C, yielding PAP-OH and 4-TPP-butan-1-ol iodide with a $t_{1/2}$ of about 17 h (data not shown).

The new derivatives were first screened for their cytotoxic activity on murine CTLL-2 lymphocytes. These cells do not express Kv1.3, and were either transfected with an empty vector to provide a control (CTLL-2/pJK) or stably transfected with an expression vector for Kv1.3 (CTLL-2/Kv1.3) (54) (**Figure 2**). Cell survival was assessed with the MTT assay. P5TP did not represent an improvement over Psora-4 or PAP-1 (**Figure 2A**). On the other hand, PCTP proved remarkably effective and exhibited selectivity toward cells expressing Kv1.3: while viability of CTLL-2/Kv1.3 cells decreased in a dose-dependent manner, that of CTLL-2/pJK cells was only slightly affected (**Figure 2B**).

Since MDR pumps may play a crucial role in extruding drugs from cells, MTT assays were also performed in the presence of MDR inhibitors (cyclosporin H and probenecid). In this specific case, MDR inhibition sensitized CTLL-2/Kv1.3 cells to Psora-4 and PAP-1, as previously reported (36), while there were no differences in the activity observed with PCTP. This observation suggests that the positively charged compound may escape MDR action due to a rapid "electrophoretic" transport through the plasma membrane, due to the negative-inside electrical potential difference. This has been already proposed for mitoVES. Contrary to VES, mitoVES was not a substrate for the ABCA1 pump in non-small cell lung carcinoma H1299 cells (55).

We then tested PCTP on two human leukemic cell lines: Jurkat leukemia T cells and K562 chronic myelogenous leukemia cells (**Figure 3A**). Cell death was determined by Annexin-V-FITC staining and FACS analysis. As expected, PCTP induced apoptosis only in Kv1.3-expressing Jurkat cells (54), while it was quite ineffective in killing K562 cells, which lack Kv1.3 (34, 56).

To further demonstrate Kv1.3 involvement in apoptosis induction by PCTP, Jurkat cells were transiently transfected with siRNA targeting Kv1.3 to reduce its expression (36). These cells have the peculiarity that they express only Kv1.3 among the potassium channels of the Kv family (54). Experiments confirmed that Kv1.3 expression is crucial for cell death induction by PCTP, since Kv1.3 silencing protected the cells from death (**Figure 3B**).

We proceeded testing PCTP also with other Kv1.3-expressing cancer cell lines. We took advantage of a mouse B16F10 melanoma cell line (**Figure 4A**), which also expresses Kv1.3 in mitochondria, as we have shown before (36). In this case the presence of MDR inhibitors was crucial, as already observed with PAP-1. Nevertheless, PCTP is more powerful than the precursor in triggering cell death.

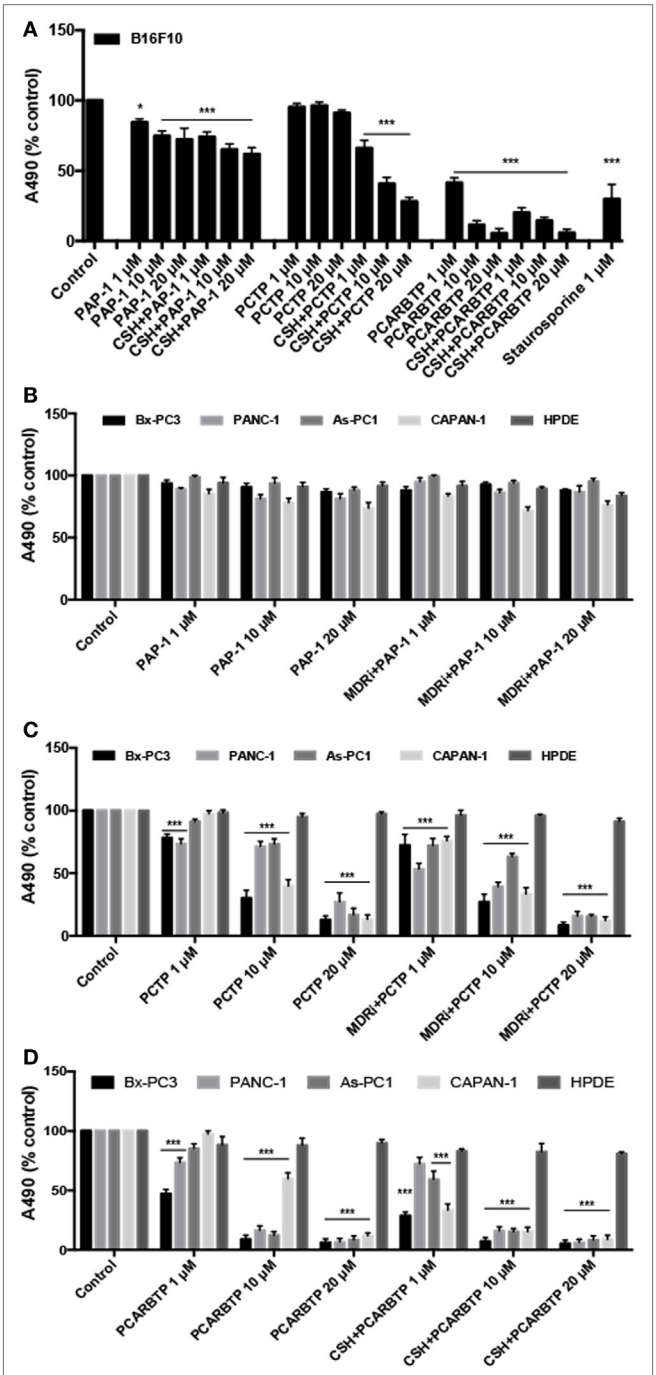


FIGURE 4 | PCTP reduces the viability of Kv1.3-expressing cells. **(A)** Mouse melanoma B16F10 cells were treated either with PAP-1, PCTP, or PCARBTP for 24 h, with or without 4 μM cyclosporine H (CSH) as multidrug resistance pumps inhibitor. Results are reported as mean percentage of viable cells normalized with respect to untreated cells ($n = 3$; $^{*}p < 0.05$; $^{***}p < 0.001$, vs control). **(B,C)** Four human pancreatic ductal adenocarcinoma cell lines (Bx-PC3, PANC-1, As-PC1, CAPAN-1) and a non tumoral human pancreatic duct epithelial line (HPDE, used as a negative control) were treated for 24 h with PAP-1 **(B)**, PCTP **(C)**, or PCARBTP **(D)**, both in absence or presence of 4 μM CSH. Mean percentage of viable cells normalized with respect to untreated cells ($n = 3$; $^{***}p < 0.001$ vs control).

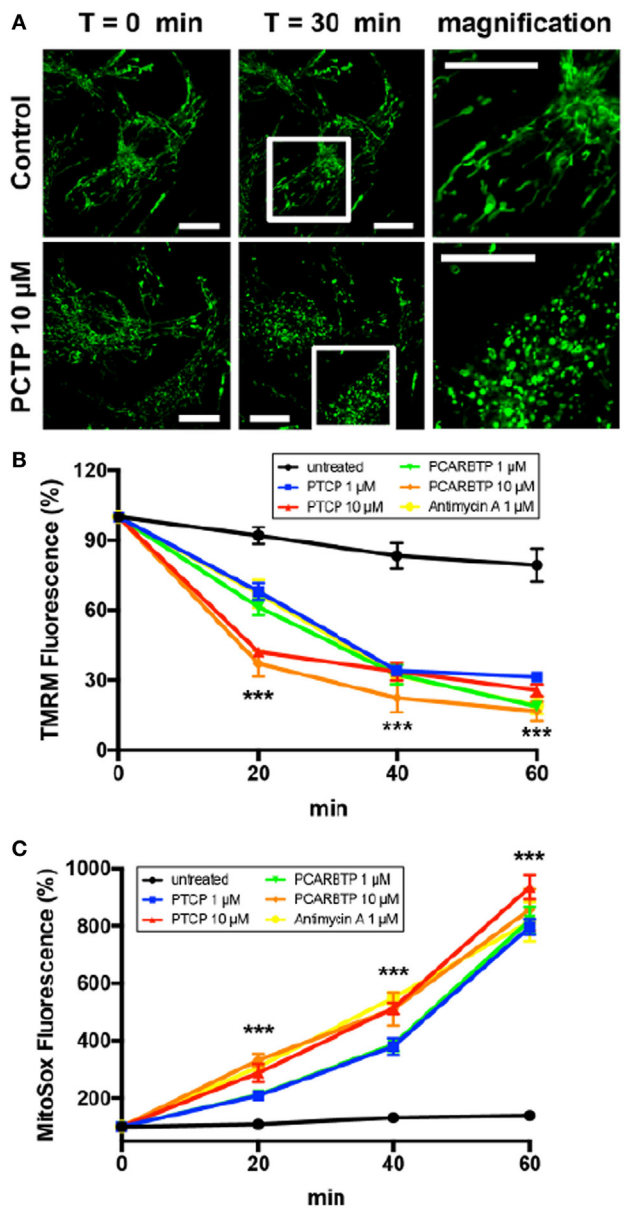


FIGURE 5 | PCTP induces mitochondrial swelling, decrease in membrane potential, and increase in ROS production in Kv1.3-expressing cells. **(A)** Mitochondrial morphology was observed in B16F10 cells after staining the mitochondrial network by incubation for 20 min at 37°C with 500 nM Mitotracker green. The effects on mitochondria have been observed by confocal microscopy after 30 min of incubation either with or without 10 μM PCTP. The images are representative of three independent experiments ($n = 3$; Bars = 15 μm). **(B)** Mitochondrial membrane potential was measured by FACS analysis of TMRM fluorescence in leukemic Jurkat T cells. Values are reported as percentage of the initial fluorescence ($n = 3$; $^{***}p < 0.001$ with respect to untreated, all other conditions). **(C)** Mitochondrial ROS production was measured by FACS analysis of the increase in the fluorescence of MitoSOX in leukemic Jurkat T cells. Values are reported as percentage of the initial fluorescence ($n = 3$; $^{***}p < 0.001$ with respect to untreated, all other conditions).

The derivative was finally tested on various PDAC cell lines, which have been shown to express Kv1.3 (51). All have been previously characterized, and were found to be mutated in p53, to express variable but robust levels of Bcl2-family anti-apoptotic proteins and to be largely resistant to standard chemotherapeutics (57–59). Most of them (with exception of Bx PC-3 cells) are also mutated in K-ras (57). These cell lines provide an *in vitro* model of one of the most feared and untreatable human cancers, for which a viable pharmacological approach is much needed.

Interestingly, also in this case PCTP proved remarkably effective in inducing cell death (**Figure 4C**) while its precursor, PAP-1, was essentially inactive (**Figure 4B**). Cytotoxicity varied somewhat from cell line to cell line (**Figure 4C**). PCTP was confirmed to induce apoptosis (not shown). These results again show that mitochondriotropic mitoKv1.3 inhibitors can overcome chemoresistance, exerting their cytotoxic effects despite alterations of the cellular anti-apoptotic apparatus.

We investigated the impact of PCTP on the mitochondria of intact cells. To monitor morphological changes we used B16F10 cells labeled with the permanent mitochondrial marker MitoTracker Green (**Figure 5A**). The exemplary images in **Figure 5A** show that the mitochondrial network underwent fragmentation. Mitochondrial fission has been firmly associated with the process of apoptosis (60–62). Mitochondrial depolarization (TMRM staining), and ROS generation (MitoSOX™ Red staining) were observed using Jurkat cells in FACS experiments (**Figures 5B,C**, respectively).

These observations are fully coherent with the mechanistic model deduced from the data obtained studying apoptosis (32) and using PAPTP to induce it (50): the initial event is channel inhibition, with consequent production of ROS. In turn, ROS promote the onset of the permeability transition, resulting in mitochondrial depolarization and further ROS release. The effects of PCTP on cancerous cells *in vitro* are comparable to those of PAPT and PCARBTP [**Figures 4** and **5**; (50)]. PCTP might conceivably even outperform these latter compounds *in vivo*, depending on factors such as pharmacokinetics and the rate of hydrolysis of the carbonate bond system. The mitochondrial effects suggest that the compound might have significant undesirable effects on healthy cells. It is, however, of relevance that non-tumoral, fast-growing, Kv1.3-expressing HPDE (51, 63) were not affected by PCTP (**Figure 4C**), as was the case also for PCARBTP (**Figure 4D**). While *in vivo* work is needed to investigate this crucial point, this observation suggests that PCTP might resemble PAPTP and PCARBTP in acting specifically on cancerous cells, sparing others.

CONCLUSION

After PCARB, we have identified another mitochondriotropic prodrug of PAP-OH, PCTP, with marked pro-apoptotic effects on Kv1.3-expressing cancerous cells, including four PDAC lines. As is also the case for other mitochondriotropic psoralenic derivatives, its administration *in vitro* causes mitochondrial dysfunction and ROS generation. The results definitely warrant further testing in *in vivo* oncological models.

AUTHOR CONTRIBUTIONS

AMat, MR, AMan, LB, and LL performed experiments. AMat, LL, IS, MZ, and CP designed research. LL, IS, MZ, and CP analyzed results. LL, LB, MZ, AMat, and IS wrote the article.

ACKNOWLEDGMENTS

The authors are grateful to Prof. H. Kalthoff for the gift of cell lines.

FUNDING

The work was supported by the Italian Association for Cancer Research (AIRC IG grant 15544 to IS), by the Italian Ministry of University and Education (PRIN 20107Z8XBW_004 to MZ, PRONAT project to MZ and LB; PRIN 2015795S5W to IS), and by the CNR (Project of Special Interest on Aging and InterOmics; to MZ and LB). LL is the recipient of a young researcher grant of the University of Padova (no. GRIC12NN5G) and a PRID 2017 (no. BIRD162511) from the University of Padova. LL is grateful to EMBO for a short-term fellowship (ASTF 233-2014).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fonc.2018.00122/full#supplementary-material.

REFERENCES

1. Moloney JN, Cotter TG. ROS signalling in the biology of cancer. *Semin Cell Dev Biol* (2018). doi:10.1016/j.semcdb.2017.05.023
2. Trachootham D, Lu W, Ogasawara MA, Nilsa RD, Huang P. Redox regulation of cell survival. *Antioxid Redox Signal* (2008) 10(8):1343–74. doi:10.1089/ars.2007.1957
3. Baracca A, Chiaradonna F, Sgarbi G, Solaini G, Alberghina L, Lenaz G. Mitochondrial complex I decrease is responsible for bioenergetic dysfunction in K-ras transformed cells. *Biochim Biophys Acta* (2010) 1797(2):314–23. doi:10.1016/j.bbabio.2009.11.006
4. Hu Y, Lu W, Chen G, Wang P, Chen Z, Zhou Y, et al. K-ras(G12V) transformation leads to mitochondrial dysfunction and a metabolic switch from oxidative phosphorylation to glycolysis. *Cell Res* (2012) 22(2):399–412. doi:10.1038/cr.2011.145
5. Kim EM, Jung CH, Kim J, Hwang SG, Park JK, Um HD. The p53/p21 complex regulates cancer cell invasion and apoptosis by targeting Bcl-2 family proteins. *Cancer Res* (2017) 77(11):3092–100. doi:10.1158/0008-5472.CAN-16-2098
6. Kim EM, Park JK, Hwang SG, Kim WJ, Liu ZG, Kang SW, et al. Nuclear and cytoplasmic p53 suppress cell invasion by inhibiting respiratory complex-I activity via Bcl-2 family proteins. *Oncotarget* (2014) 5(18):8452–65. doi:10.18632/oncotarget.2320
7. Kim EM, Kim J, Park JK, Hwang SG, Kim WJ, Lee WJ, et al. Bcl-w promotes cell invasion by blocking the invasion-suppressing action of Bax. *Cell Signal* (2012) 24(6):1163–72. doi:10.1016/j.cellsig.2012.01.019
8. Um HD. Bcl-2 family proteins as regulators of cancer cell invasion and metastasis: a review focusing on mitochondrial respiration and reactive oxygen species. *Oncotarget* (2016) 7(5):5193–203. doi:10.18632/oncotarget.6405
9. Ciccarese F, Ciminale V. Escaping death: mitochondrial redox homeostasis in cancer cells. *Front Oncol* (2017) 7:117. doi:10.3389/fonc.2017.00117
10. Galadari S, Rahman A, Pallichankandy S, Thayyullathil F. Reactive oxygen species and cancer paradox: to promote or to suppress? *Free Radic Biol Med* (2017) 104:144–64. doi:10.1016/j.freeradbiomed.2017.01.004
11. Zou Z, Chang H, Li H, Wang S. Induction of reactive oxygen species: an emerging approach for cancer therapy. *Apoptosis* (2017) 22(11):1321–35. doi:10.1007/s10495-017-1424-9
12. Redza-Dutordoir M, Averill-Bates DA. Activation of apoptosis signalling pathways by reactive oxygen species. *Biochim Biophys Acta* (2016) 1863(12):2977–92. doi:10.1016/j.bbamcr.2016.09.012
13. Sakauchi C, Wakatsuki H, Ichijo H, Hattori K. Pleiotropic properties of ASK1. *Biochim Biophys Acta* (2017) 1861(1 Pt A):3030–8. doi:10.1016/j.bbagen.2016.09.028
14. Vanlangenakker N, Vanden Berghe T, Krysko DV, Festjens N, Vandenabeele P. Molecular mechanisms and pathophysiology of necrotic cell death. *Curr Mol Med* (2008) 8(3):207–20. doi:10.2174/156652408784221306
15. Stockwell BR, Friedmann Angeli JP, Bayir H, Bush AI, Conrad M, Dixon SJ, et al. Ferroptosis: a regulated cell death nexus linking metabolism, redox biology, and disease. *Cell* (2017) 171(2):273–85. doi:10.1016/j.cell.2017.09.021
16. Kong H, Chandel NS. Regulation of redox balance in cancer and T cells. *J Biol Chem* (2018). doi:10.1074/jbc.TM117.000257
17. Ding C, Fan X, Wu G. Peroxiredoxin 1 – an antioxidant enzyme in cancer. *J Cell Mol Med* (2017) 21(1):193–202. doi:10.1111/jcmm.12955
18. Yang YJ, Baek JY, Goo J, Shin Y, Park JK, Jang JY, et al. Effective killing of cancer cells through ROS-mediated mechanisms by AMRI-59 targeting peroxiredoxin I. *Antioxid Redox Signal* (2016) 24(8):453–69. doi:10.1089/ars.2014.6187
19. Sassi N, Biasutto L, Mattarei A, Carraro M, Giorgio V, Citta A, et al. Cytotoxicity of a mitochondriotropic quercetin derivative: mechanisms. *Biochim Biophys Acta* (2012) 1817(7):1095–106. doi:10.1016/j.bbabio.2012.03.007
20. Sassi N, Mattarei A, Azzolini M, Szabo I, Paradisi C, Zoratti M, et al. Cytotoxicity of mitochondria-targeted resveratrol derivatives: interactions with respiratory chain complexes and ATP synthase. *Biochim Biophys Acta* (2014) 1837(10):1781–9. doi:10.1016/j.bbabio.2014.06.010
21. Chen Y, Zhang H, Zhou HJ, Ji W, Min W. Mitochondrial redox signaling and tumor progression. *Cancers* (2016) 8(4):40. doi:10.3390/cancers8040040
22. Dong LF, Kovarova J, Bajzikova M, Bezawork-Geleta A, Svec D, Endaya B, et al. Horizontal transfer of whole mitochondria restores tumorigenic potential in mitochondrial DNA-deficient cancer cells. *Elife* (2017) 6:e22187. doi:10.7554/eLife.22187
23. Porporato PE, Filigheddu N, Bravo-San Pedro JM, Kroemer G, Galluzzi L. Mitochondrial metabolism and cancer. *Cell Res* (2018) 28(3):265–80. doi:10.1038/cr.2017.155
24. Sciacovelli M, Gaude E, Hilvo M, Frezza C. The metabolic alterations of cancer cells. *Methods Enzymol* (2014) 542:1–23. doi:10.1016/B978-0-12-416618-9.00001-7
25. Tan AS, Baty JW, Dong LF, Bezawork-Geleta A, Endaya B, Goodwin J, et al. Mitochondrial genome acquisition restores respiratory function and tumorigenic potential of cancer cells without mitochondrial DNA. *Cell Metab* (2015) 21(1):81–94. doi:10.1016/j.cmet.2014.12.003
26. Viale A, Pettazzoni P, Lyssiotis CA, Ying H, Sanchez N, Marchesini M, et al. Oncogene ablation-resistant pancreatic cancer cells depend on mitochondrial function. *Nature* (2014) 514(7524):628–32. doi:10.1038/nature13611
27. Biasutto L, Dong LF, Zoratti M, Neuzil J. Mitochondrially targeted anti-cancer agents. *Mitochondrion* (2010) 10(6):670–81. doi:10.1016/j.mito.2010.06.004
28. Fulda S, Galluzzi L, Kroemer G. Targeting mitochondria for cancer therapy. *Nat Rev Drug Discov* (2010) 9(6):447–64. doi:10.1038/nrd3137
29. Yan B, Dong L, Neuzil J. Mitochondria: an intriguing target for killing tumour-initiating cells. *Mitochondrion* (2016) 26:86–93. doi:10.1016/j.mito.2015.12.007
30. Szabo I, Zoratti M. Mitochondrial channels: ion fluxes and more. *Physiol Rev* (2014) 94(2):519–608. doi:10.1152/physrev.00021.2013
31. Leanza L, Zoratti M, Gulbins E, Szabo I. Mitochondrial ion channels as oncological targets. *Oncogene* (2014) 33(49):5569–81. doi:10.1038/onc.2013.578
32. Szabo I, Bock J, Grassme H, Soddemann M, Wilker B, Lang F, et al. Mitochondrial potassium channel Kv1.3 mediates Bax-induced apoptosis in lymphocytes. *Proc Natl Acad Sci U S A* (2008) 105(39):14861–6. doi:10.1073/pnas.0804236105
33. Szabo I, Soddemann M, Leanza L, Zoratti M, Gulbins E. Single-point mutations of a lysine residue change function of Bax and Bcl-xL expressed in Bax- and Bak-less mouse embryonic fibroblasts: novel insights into the molecular mechanisms of Bax-induced apoptosis. *Cell Death Differ* (2011) 18(3):427–38. doi:10.1038/cdd.2010.112

34. Leanza L, O'Reilly P, Doyle A, Venturini E, Zoratti M, Szegezdi E, et al. Correlation between potassium channel expression and sensitivity to drug-induced cell death in tumor cell lines. *Curr Pharm Des* (2014) 20(2):189–200. doi:10.2174/13816128113199990032
35. Vennekamp J, Wulff H, Beeton C, Calabresi PA, Grissmer S, Hansel W, et al. Kv1.3-blocking 5-phenylalkoxypsoralens: a new class of immunomodulators. *Mol Pharmacol* (2004) 65(6):1364–74. doi:10.1124/mol.65.6.1364
36. Leanza L, Henry B, Sassi N, Zoratti M, Chandy KG, Gulbins E, et al. Inhibitors of mitochondrial Kv1.3 channels induce Bax/Bak-independent death of cancer cells. *EMBO Mol Med* (2012) 4(7):577–93. doi:10.1002/emmm.201200235
37. Leanza L, Trentin L, Becker KA, Frezzato F, Zoratti M, Semenzato G, et al. Clofazimine, Psora-4 and PAP-1, inhibitors of the potassium channel Kv1.3, as a new and selective therapeutic strategy in chronic lymphocytic leukemia. *Leukemia* (2013) 27(8):1782–5. doi:10.1038/leu.2013.56
38. Guzman-Villanueva D, Weissig V. Mitochondria-targeted agents: mitochondriotropics, mitochondriotoxics, and mitocans. *Handb Exp Pharmacol* (2017) 240:423–38. doi:10.1007/164_2016_37
39. Kalyanaraman B, Cheng G, Hardy M, Ouari O, Lopez M, Joseph J, et al. A review of the basics of mitochondrial bioenergetics, metabolism, and related signaling pathways in cancer cells: therapeutic targeting of tumor mitochondria with lipophilic cationic compounds. *Redox Biol* (2018) 14:316–27. doi:10.1016/j.redox.2017.09.020
40. Murphy MP, Smith RA. Targeting antioxidants to mitochondria by conjugation to lipophilic cations. *Annu Rev Pharmacol Toxicol* (2007) 47:629–56. doi:10.1146/annurev.pharmtox.47.120505.105110
41. Zielonka J, Joseph J, Sikora A, Hardy M, Ouari O, Vasquez-Vivar J, et al. Mitochondria-targeted triphenylphosphonium-based compounds: syntheses, mechanisms of action, and therapeutic and diagnostic applications. *Chem Rev* (2017) 117(15):10043–120. doi:10.1021/acs.chemrev.7b00042
42. Dong LF, Jameson VJ, Tilly D, Cerny J, Mahdavian E, Marin-Hernandez A, et al. Mitochondrial targeting of vitamin E succinate enhances its pro-apoptotic and anti-cancer activity via mitochondrial complex II. *J Biol Chem* (2011) 286(5):3717–28. doi:10.1074/jbc.M110.186643
43. Kluckova K, Bezawork-Geleta A, Rohlena J, Dong L, Neuzil J. Mitochondrial complex II, a novel target for anti-cancer agents. *Biochim Biophys Acta* (2013) 1827(5):552–64. doi:10.1016/j.bbabio.2012.10.015
44. Rodriguez-Enriquez S, Hernandez-Esquivel L, Marin-Hernandez A, Dong LF, Akporiaye ET, Neuzil J, et al. Molecular mechanism for the selective impairment of cancer mitochondrial function by a mitochondrially targeted vitamin E analogue. *Biochim Biophys Acta* (2012) 1817(9):1597–607. doi:10.1016/j.bbabio.2012.05.005
45. Boukalova S, Stursa J, Werner L, Ezrova Z, Cerny J, Bezawork-Geleta A, et al. Mitochondrial targeting of metformin enhances its activity against pancreatic cancer. *Mol Cancer Ther* (2016) 15(12):2875–86. doi:10.1158/1535-7163.MCT-15-1021
46. Cheng G, Zielonka J, Ouari O, Lopez M, McAllister D, Boyle K, et al. Mitochondria-targeted analogues of metformin exhibit enhanced antiproliferative and radiosensitizing effects in pancreatic cancer cells. *Cancer Res* (2016) 76(13):3904–15. doi:10.1158/0008-5472.CAN-15-2534
47. Rohlenova K, Sachaphibulkij K, Stursa J, Bezawork-Geleta A, Blecha J, Endaya B, et al. Selective disruption of respiratory supercomplexes as a new strategy to suppress Her2(high) breast cancer. *Antioxid Redox Signal* (2017) 26(2):84–103. doi:10.1089/ars.2016.6677
48. Biasutto L, Mattarei A, Marotta E, Bradaschia A, Sassi N, Garbisa S, et al. Development of mitochondria-targeted derivatives of resveratrol. *Bioorg Med Chem Lett* (2008) 18(20):5594–7. doi:10.1016/j.bmcl.2008.08.100
49. Mattarei A, Biasutto L, Marotta E, De Marchi U, Sassi N, Garbisa S, et al. A mitochondriotropic derivative of quercetin: a strategy to increase the effectiveness of polyphenols. *Chembiochem* (2008) 9(16):2633–42. doi:10.1002/cbic.200800162
50. Leanza L, Romio M, Becker KA, Azzolini M, Trentin L, Managò A, et al. Direct pharmacological targeting of a mitochondrial ion channel selectively kills tumor cells in vivo. *Cancer Cell* (2017) 31(4):516–31.e10. doi:10.1016/j.ccell.2017.03.003
51. Zaccagnino A, Manago A, Leanza L, Gontarewitz A, Linder B, Azzolini M, et al. Tumor-reducing effect of the clinically used drug clofazimine in a SCID mouse model of pancreatic ductal adenocarcinoma. *Oncotarget* (2017) 8(24):38276–93. doi:10.18632/oncotarget.11299
52. Ouyang H, Mou Lj, Luk C, Liu N, Karaskova J, Squire J, et al. Immortal human pancreatic duct epithelial cell lines with near normal genotype and phenotype. *Am J Pathol* (2000) 157(5):1623–31. doi:10.1016/S0002-9440(10)64800-6
53. Zimin PI, Garic B, Bodendiek SB, Mahieux C, Wulff H, Zhorov BS. Potassium channel block by a tripartite complex of two cationophilic ligands and a potassium ion. *Mol Pharmacol* (2010) 78(4):588–99. doi:10.1124/mol.110.064014
54. Szabo I, Bock J, Jekle A, Soddemann M, Adams C, Lang F, et al. A novel potassium channel in lymphocyte mitochondria. *J Biol Chem* (2005) 280(13):12790–8. doi:10.1074/jbc.M413548200
55. Prochazka L, Koudelka S, Dong LF, Stursa J, Goodwin J, Neca J, et al. Mitochondrial targeting overcomes ABCA1-dependent resistance of lung carcinoma to alpha-tocopheryl succinate. *Apoptosis* (2013) 18(3):286–99. doi:10.1007/s10495-012-0795-1
56. Smith GA, Tsui HW, Newell EW, Jiang X, Zhu XP, Tsui FW, et al. Functional up-regulation of HERG K+ channels in neoplastic hematopoietic cells. *J Biol Chem* (2002) 277(21):18528–34. doi:10.1074/jbc.M200592200
57. Monti P, Marchesi F, Reni M, Mercalli A, Sordi V, Zerbi A, et al. A comprehensive in vitro characterization of pancreatic ductal carcinoma cell line biological behavior and its correlation with the structural and genetic profile. *Virchows Arch* (2004) 445(3):236–47. doi:10.1007/s00428-004-1053-x
58. Sipos B, Moser S, Kalthoff H, Torok V, Lohr M, Kloppel G. A comprehensive characterization of pancreatic ductal carcinoma cell lines: towards the establishment of an in vitro research platform. *Virchows Arch* (2003) 442(5):444–52. doi:10.1007/s00428-003-0784-4
59. Ungefroren H, Voss M, Jansen M, Roeder C, Henne-Bruns D, Kremer B, et al. Human pancreatic adenocarcinomas express Fas and Fas ligand yet are resistant to Fas-mediated apoptosis. *Cancer Res* (1998) 58(8):1741–9.
60. Aouacheria A, Baghdiguian S, Lamb HM, Huska JD, Pineda FJ, Hardwick JM. Connecting mitochondrial dynamics and life-or-death events via Bcl-2 family proteins. *Neurochem Int* (2017) 109:141–61. doi:10.1016/j.neuint.2017.04.009
61. Morciano G, Pedriali G, Sbano L, Iannitti T, Giorgi C, Pinton P. Intersection of mitochondrial fission and fusion machinery with apoptotic pathways: role of Mcl-1. *Biol Cell* (2016) 108(10):279–93. doi:10.1111/boc.201600019
62. Scorrano L. Keeping mitochondria in shape: a matter of life and death. *Eur J Clin Invest* (2013) 43(8):886–93. doi:10.1111/eci.12135
63. Liu N, Furukawa T, Kobari M, Tsao MS. Comparative phenotypic studies of duct epithelial cell lines derived from normal human pancreas and pancreatic carcinoma. *Am J Pathol* (1998) 153(1):263–9. doi:10.1016/S0002-9440(10)65567-8

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer VC declared a shared affiliation, with no collaboration, with the authors to the handling Editor.

frontiers in Oncology

HYPOTHESIS AND THEORY
published: 08 May 2018
doi: 10.3389/fonc.2018.00148

Pushing the Limits of Cancer Therapy: The Nutrient Game

Daniele Lettieri-Barbato and Katia Aquilano*

Department of Biology, University of Rome Tor Vergata, Rome, Italy

The standard cancer treatments include chemotherapy, radiotherapy, or their combination, which are generally associated with a multitude of side effects ranging from discomfort to the development of secondary tumors and severe toxicity to multiple systems including immune system. Mounting evidence has highlighted that the fine-tuning of nutrients may selectively sensitize cancer cells to conventional cancer therapies, while simultaneously protecting normal cells from their side effects. Nutrient modulation through diet also improves cancer immunesurveillance in a way that severe immunosuppression could be avoided or even the immune response or immune-based cancer therapies be potentiated also through patient microbiota remodeling. Here, we review recent advances in cancer therapy focusing on the effects of adjuvant dietary interventions (e.g., ketogenic diets, fasting) on the metabolic pathways within cancer cells and tumor environment (e.g., microbiota, immune system, tumor microenvironment) that are involved in cancer progression and resistance as well as cancer cell death. Finally, based on the overall literature data, we designed a nutritional intervention consisting in a plant-based moderate ketogenic diet that could be exploited for future preclinical research in cancer therapy.

Keywords: mitochondria, diet, fasting, immunemodulation, microbiota and immunity

OPEN ACCESS

Edited by:
Salvatore Rizza, Danish Cancer Society, Denmark

Reviewed by:
Colin E. Champ, University of Pittsburgh Cancer Institute, United States
Enrico Desideri, Universität Wien, Austria

****Correspondence:***
Daniele Lettieri-Barbato
d.lettieribarbato@hotmail.it

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *12 February 2018*
Accepted: *23 April 2018*
Published: *08 May 2018*

Citation:
Lettieri-Barbato D and Aquilano K (2018) Pushing the Limits of Cancer Therapy: The Nutrient Game. Front. Oncol. 8:148. doi: 10.3389/fonc.2018.00148

AN OVERVIEW ON THE CONTROL OF TUMOR PROGRESSION BY DIETARY INTERVENTIONS

A plethora of epidemiological and experimental data demonstrated the efficacy of geroprotective dietary regimens (e.g., fasting, calorie, proteins, or single amino acids restrictions) in cancer prevention (1–3). Furthermore, such dietary patterns are emerging to be effective in selectively killing cancer cells, whereas increasing resistance of normal cells to the toxic effects of the anticancer therapeutics.

Calorie restriction (CR), defined as 30–60% less of daily calorie requirement without malnutrition, is known to extend healthy life span from yeast to mammals (4). The anticancer effects of CR are known since several years (5). CR is particularly effective in reducing the incidence, mass, and metastasis of breast cancer cells (6, 7). Remarkably, applying CR in combination with radiotherapy enhanced the radiotherapy efficacy inducing a more pronounced apoptosis of breast cancer cells than radiotherapy alone (7). In human, however, CR requires high compliance challenges to be maintained for adequate therapeutic period. For these reasons, short period of fasting without malnutrition have been proposed as potentially safe interventions to be associated with cancer treatments (8).

Fasting is commonly defined as a time-controlled deprivation of all kinds of foods and dietary nutrients. Differently to nocturnal fasting, time-controlled fasting leads to a profound metabolic reprogramming building up adaptive stress responses that are involved in life and health span

extension (9–13). However, the adaptive stress responses induced by fasting occurring in normal cells differ from those activated by cancer cells because oncogenes might limit the activation of nutrient-sensing pathways while increasing chemotherapy vulnerability (8). Notably, proto-oncogenes such as IGF1R, PI3K, and AKT activate growth signaling and addict cancer cells to nutrient such as glucose and amino acids to meet their high proliferative rate (8). It has been shown that different cycles of fasting are effective in limiting tumor progression in several murine cancer models (14–17). However, the greatest effects were observed when fasting was combined with the conventional chemotherapy or radiotherapy (14–18). Interestingly, in these studies, fasting interventions alone do not cause clear signs of discomfort, but rather improve the animal condition. When fasting was combined with conventional therapies (e.g., temozolomide), most of the mice appeared healthy with the tumor-size below the controls, indicating that the combination of both treatments is well tolerated and improve tumor-bearing survival (14). The protective role of fasting against the side effects of anticancer therapy was confirmed in another study in which fasting was able to improve the overall cardiac response (maintenance of diastolic/systolic volumes and left ventricle wall thickness) to high-dose of doxorubicin (19). Fasting also exerted a significant protection against reduced mobility, ruffled hair, and hunched back posture caused by high dose of etoposide in mice (20). The anticancer effects of fasting might also rely on ketone bodies increase (21, 22). In support of this assumption, meta-analysis on ketogenic diets (KD), low in carbohydrates and high in fats, suggested a salutary impact on survival in animal models, with benefits prospectively linked to the magnitude of ketosis, time of diet initiation, and tumor location (23). Other evidence also demonstrated that KD might be safely used as adjuvant therapies to conventional radiation and chemotherapies (24). In particular, KD together with conventional radiotherapy led to increased radiation sensitivity in pancreatic cancer xenografts in mice (25). Similar results were obtained in mice bearing lung cancer xenografts (26). However, patients have demonstrated difficulty to comply with a KD while receiving concurrent radiation and chemotherapy in advanced lung and pancreatic cancer (25). Therefore, as better tolerated with respect to CR and KD, fasting appears to be more promising as adjuvant treatment in cancer therapy. Finally, it has been demonstrated that fasting could be replaced by the administration of CR mimetics, which showed the capability to improve the efficacy of chemotherapy as well. However, the objective response rates with metformin (27–30) or rapalogs (31) in clinical trials are still unclear and comparative analyses delineating a selective effectiveness of these drugs in cancer treatment and patient tolerability have to be more deeply elucidated.

NUTRIENT MODULATION IN PROLIFERATING/RESILIENT CANCER CELLS: A MOLECULAR VIEW

The reduced levels of nutrients and growth factors observed during fasting led to hypothesize their mandatory role in governing the differential stress responses in normal and cancer cells (10, 14, 16, 18). The different responses of normal and cancer cells to fasting shed light on their different sensitivity to nutrients and growth factors (18).

IGF-1/IGF-1R signaling is strongly dependent on nutrient availability and involves intensification of cancer cell proliferation, through the direct effects on PI3K/Akt signaling, and resistance to cell death imposed by chemotherapeutics and radiotherapy (**Figure 1**) (32). Indeed, fasting reduces circulating IGF-1 levels and this event protects mice deficient in the liver production of IGF-1 against chemotherapy drugs (16). Accordingly, restoration of IGF-1 was sufficient to reverse the protective effect of fasting (16). Reducing IGF-1 protects primary glia, but not glioma cells, against cyclophosphamide and mouse embryonic fibroblasts against doxorubicin (16). In the opposite manner, IGF-1 supplementation in starved breast cancer cells reversed drug sensitization. Overall, these findings strongly indicate that the fasting-mediated sensitization of cancer cells to chemotherapeutic drugs is conferred by the decrease of IGF-1 levels (15).

Nutrient shortage *per se* is able to increase mitochondrial reactive oxygen species (ROS) production in cancer cells arguing that limiting nutrient availability could enhance the effectiveness of redox-based cancer therapeutics (**Figure 1**) (33, 34). Actually, in breast cancer and melanoma cells, nutrient starvation was found to increase superoxide levels and aggravate oxidative stress caused by cyclophosphamide and cisplatin (15, 35). When applied in combination, fasting and chemotherapy act in synergy in elevating ROS levels and triggering DNA damage also in *in vivo* models of cancer (36). Micro-PET analyses in murine models of colon cancer cells revealed that fasting is effective as oxaliplatin (OXP) in reducing the average tumor glucose consumption and the lowest values were achieved by coupling fasting with OXP. In colon cancer cells, nutrient starvation upregulates oxidative phosphorylation with a significant production in mitochondrial superoxide caused by electron leakage. Consequently, starvation or OXP alone markedly increased ROS generation and their combination (starvation plus OXP) exacerbated ROS production in colon cancer cells (36). The hypothesis that cytotoxicity induced by glucose deprivation in cancer cells is mediated by mitochondrial superoxide and H_2O_2 was confirmed by exposing glucose-deprived transformed human fibroblasts to electron transport chain blockers (ETCBs), known to increase mitochondrial superoxide and H_2O_2 production (37). Glucose deprivation in the presence of ETCBs enhanced oxidative stress as well as cell death in several different human cancer cell lines (PC-3, DU145, MDA-MB231, and HT-29). In addition, human osteosarcoma cells lacking functional mitochondrial electron transport chain [rho(0)] were resistant to glucose deprivation-induced cytotoxicity and oxidative stress in the presence of antimycin A (complex III inhibitor), thus highlighting the role of mitochondrial ROS as mediators of cancer cell death (37).

The mechanisms by which KDs act as adjuvants in cancer therapy also seem to be associated with increased oxidative stress within cancer cells (24). Indeed, upon KD, the high level of circulating fatty acids limits the availability of glucose for glycolysis (Randle's Cycle) (38). This reduces the formation of pyruvate and glucose-6-phosphate and in turn the synthesis of

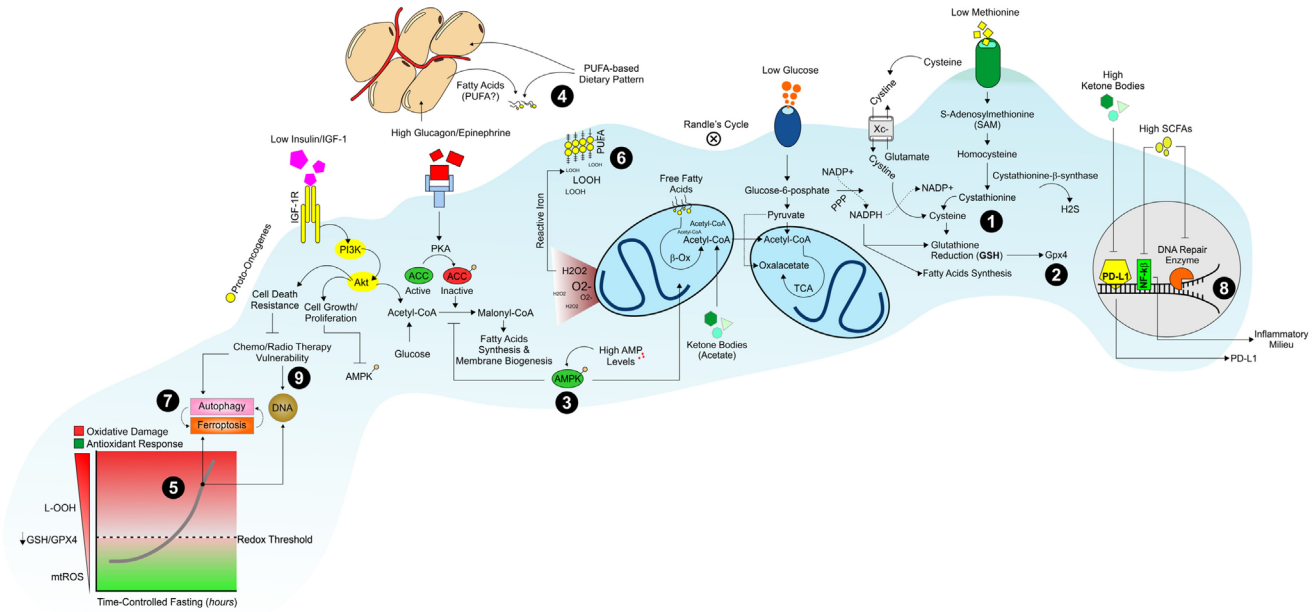


FIGURE 1 | Dietary strategy to promote a hostile metabolism in proliferating/resilient cancer cells. Dietary patterns low in proteins, starch, and sugars promote an environment poor in sulfur amino acids (i.e., methionine, cysteine), glucose, and growth factors (insulin/IGF-1) that could limit NADPH/GSH production (1) and GPX4 activity (2). The diminished levels of glucose and glucagon/insulin ratio switch-off lipid synthesis and switch-on AMPK-driven lipid oxidation pathways in proliferating cancer cells (3). Under such metabolic conditions, cancer cells build-up their membranes by using extracellular dietary-derived and/or white adipose tissues-released fatty acids (4). The concomitant activation of OxPHOS metabolism and reduction in GSH levels are causative of oxidative stress (5) culminating in a massive lipid peroxidation (LOOH) (6) and ferroptosis in cancer cells (7). Diet low in starch, sugars, and proteins but rich in fatty acids also increases ketone bodies and modulates gut microbiota features by producing short-chain fatty acids (SCFAs). Ketone bodies and SCFAs affect PD-L1, nuclear factor-kb, and DNA repair enzymes genes transcription (8) promoting the chemo/radiotherapy vulnerability of cancer cells (9).

NADPH through the pentose phosphate pathway (PPP) (39). NADPH is necessary for buffering hydroperoxides (LOOH) production *via* the NADPH-dependent glutathione/glutathione peroxidase (GSH/GPX) system (40, 41). As consequence, an increase of LOOH is likely elicited (24) (**Figure 1**). Accordingly, hyperketotic diabetic patients have a higher level of lipid peroxidation in erythrocytes membrane and a significant decrease in cellular GSH levels than normal ketonic diabetic patients (42). Treatments with the ketone body acetoacetate elevated the levels of lipid peroxidation in human endothelial cells inhibiting their proliferation (42). This evidence suggests a direct role of ketone bodies in directly affecting GSH levels.

The main non-enzymatic cellular antioxidant GSH acts as an electron donor to reduce oxidized macromolecules, becoming itself oxidized in the process. Oxidized glutathione (GSSG) may then be restored in GSH through the action of the NADPH-dependent glutathione reductase (43). This enzymatic process generates $NADP^+$, which may be reconverted to NADPH using electrons obtained from different biochemical pathways (44). Thus, proliferating cancer cells develop a peculiar metabolic flexibility to maintain a functional redox threshold by regulating NADPH levels through glycolytic flux modulation (33). Indeed, glucose-addicted human cancer cells cultured in a low-glucose medium without serum and amino acids are able to reprogram their metabolism by shifting toward PPP, which sustains the production of NADPH to dampen oxidative stress (33). However, during the initial stages of solid tumor development, when cells migrate to the lumen of lymphatic or blood vessels by loss of attachment (LOA) to the extracellular matrix, the glucose availability could not be sufficient to produce an adequate amount of NADPH and proliferation is inhibited (45). Upon such environmental stress, cancer cells induce adaptive responses consisting in the activation of AMPK signaling that inhibits fatty acid synthesis and triggers fatty acids oxidation to maintain energy production and NADPH levels (46, 47). Although cancer cells build up such adaptive responses, it has been observed that during LOA, cancer cells undergo ATP and NADPH drop and increase ROS production (48). Several papers demonstrated that cancer cells experiencing glucose shortage might maintain their proliferative capacity and membrane biogenesis by the uptake of extracellular lipids (49). Accordingly, extracellular saturated fatty acids supplementation supports the proliferative demand for biomass synthesis of proliferating cells (50, 51). Otherwise, supplementation with polyunsaturated fatty acids (PUFA) induced a significant cytotoxic effect on cancer cells either alone (52–54) or in combination with conventional anticancer therapies (55, 56). Differently to saturated fatty acids, PUFA are strongly susceptible to peroxidation (lipid peroxidation) in *in vivo* systems (57, 58). This appears to be a key mechanism triggering cancer cell death (59). With all this in mind, forcing the changes in the membrane lipids composition by dietary/nutrient enrichment in PUFA might promote an

intrinsic sensitivity toward lipid peroxidation (57, 58, 60) and cancer cell death (**Figure 1**).

NUTRIENT-MEDIATED COMMITMENT TO FERROPTOSIS IN CANCER CELLS

By preserving NADPH levels, cancer cells sustain GPX/GSH activity during nutrient limitation, and this may confer resistance to redox-based chemotherapeutics (61–63). Indeed, many rebel cancer cells use a common trick to evade annihilation; they enter into what is known as a mesenchymal state that is "epithelial-to-mesenchymal" transition, which provides cancer cell resistance to conventional therapeutic regimens (64). It has been demonstrated that high therapy-resistant mesenchymal cancer cells strictly rely on the selenium-dependent GPX4 for survival (65). By using the reducing power of GSH, GPX4 converts potentially toxic L-OOH to non-toxic lipid alcohols (L-OH) (**Figure 1**) (66–68). Accordingly, inactivation of GPX4 through GSH depletion with erastin, or with a direct GPX4 inhibitor, ultimately results in lipid peroxidation in cancer cells (69). It is thus provocative to hypothesize that the evolutionary pressure to maintain the selenium protein GPX4 might correlate with an organism's requirement for an increased PUFA content, which, in turn, renders complex biological activities possible (70).

Uncontrolled lipid peroxidation is causative of the onset of a metabolically regulated cell death called "ferroptosis," which is characterized by the iron-dependent formation of LOOH leading to cell death (**Figure 1**) (71). Sulfur amino acids play a key role in ferroptosis. In particular, agents that inhibit cystine uptake *via* the cystine/glutamate antiporter (XC system), such as sulfasalazine or erastin, arrest tumor growth and induce ferroptosis (72, 73). The uptake of cystine is followed by its NADPH-dependent conversion in cysteine, the rate-limiting amino acid precursor for the GSH biosynthesis (74). Direct depletion of cystine from plasma using an engineered cystine-degrading enzyme conjugate arrests tumor growth and triggers cell death (75). Agents that conjugate to GSH, as well as chemical or genetic inhibition of GSH biosynthesis, disrupt tumor cell growth and induce a ferroptosis-like form of cell death (76). Ferroptosis appears to be an effective cell death mechanism in cancer cells, since lipophilic antioxidant α-tocopherol or iron chelators, such as deferoxamine, efficiently dampen it (77). Hence, the presence of extracellular cysteine and cystine are crucial for growth and proliferation of various types of cancer, as these amino acids maintain GSH levels and prevent oxidative stress (**Figure 1**) (78–80). Because cysteine is limiting in the biosynthesis of GSH, some cancer cells, under cysteine unavailability, make use of the transsulfuration pathway to biosynthesize cysteine from methionine (Met), a dietary essential sulfur amino acid (81, 82). The essentiality of Met in cancer is supported by the evidence that some cancer cells display a higher sensitivity to Met shortage with respect to normal cells (83–87). The first steps of the transsulfuration pathway are the conversion to *S*-adenosylmethionine (SAM) and transfer of the methyl group of SAM to a large variety of methyl acceptors with formation of *S*-adenosylhomocysteine (SAH) (88), which can be then converted to homocysteine (Hcy) by SAH hydrolase (AHCY) (89). Alternatively, Hcy is converted to cystathionine by cystathionine β-synthase (CBS). CBS catalyzes the condensation of Hcy and serine, thereby forming cystathionine, which is subsequently cleaved to cysteine. Furthermore, exogenous cysteine is also essential for several cancer types (glioma, prostate, and pancreatic), as blocking its uptake through the cystine/glutamate antiporter reduces viability due to the cell death caused by uncontrolled oxidative stress (90–92). Similarly, CBS blockage reduces cancer cell proliferation and attenuates growth of patient-derived colon cancer xenografts models (93). Although these findings suggest that fasting or selective nutrient modulation could trigger ferroptotic cell death in cancer cells, a clear evidence linking nutrient availability to ferroptosis is still lacking. Several works demonstrated that starved cancer cells (mainly in amino acids) as well as cells lacking the enzyme producing NADPH from glucose (glucose-6-phosphate dehydrogenase) experience massive ROS production and autophagy-dependent cell death (33, 94, 95). Autophagy is a process described as intracellular removal of damaged organelles by self-degradative process (96). Interestingly, a tight relationship between autophagic cell death and ferroptosis is emerging (97–99). Indeed, it seems that autophagy activation leads to a degradation of ferritin (ferritinophagy) (97), thus increasing the intracellular free iron levels promoting ROS production and ferroptosis (**Figure 1**) (99).

DIETARY STRATEGIES TO BOOST THE IMMUNOMETABOLIC RESPONSES IN CANCER THERAPY

Short-term fasting has a beneficial impact on cancer immunosurveillance (100). In particular, Pietrocola and co-workers demonstrated that fasting or CR-mimicking drugs, induce the depletion of regulatory T cells (which dampen anticancer immunity), thus igniting autophagic flux in murine models of KRAS-induced lung cancers. Accordingly, the inhibitory effect of fasting on tumor growth is lost in cancers that have been rendered autophagy deficient (100). Recently, also, isocaloric diet with protein restriction has been demonstrated to induce an IRE1α-dependent UPR in cancer cells, enhancing cytotoxic $CD8^+$ T cell (a type of effector T lymphocyte)-mediated response against tumors (101).

Similarly to what observed with prolonged fasting (102), cycles of a fasting-mimicking diet (FMD) are effective in increasing hematopoietic cells proliferation and promoting immune system regeneration and modulation (103). Importantly, FMD has stimulatory effect on common lymphoid progenitor cells and $CD8^+$ T cell-dependent cytotoxicity on breast cancer and melanoma cells (**Figure 2**) (17, 102). The presence of cytotoxic $CD8^+$ T cells in the tumor environment [tumor infiltrating lymphocytes (TIL)] is considered a positive outcome of the cancer treatment (104, 105).

$CD8^+$ T cells are influenced by nutrients and other supportive signals that are generally available in their environment. Generally, tumor cells inactivate $CD8^+$ T cells. The suppression of oxidative phosphorylation and an upregulated glycolytic flux

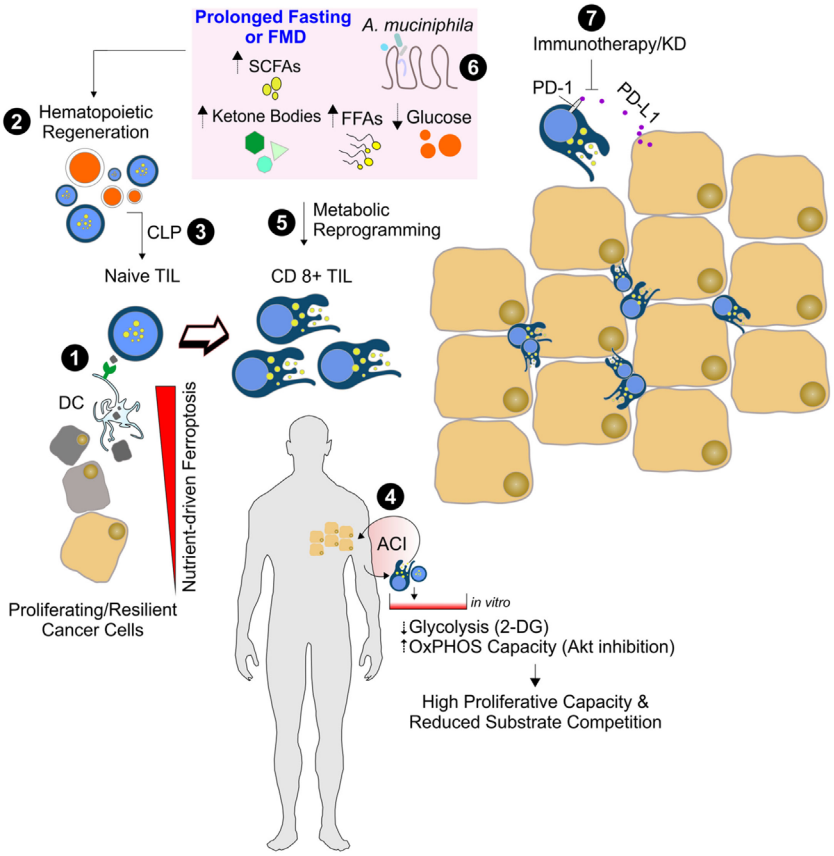


FIGURE 2 | Nutrient manipulation to boost immunometabolic phenotype of CD8⁺ tumor infiltrating lymphocytes (TILs). Naïve CD8⁺ T cells recognize the antigen of ferroptotic cancer cells on class I MHC on dendritic cells, thus becoming mature cytotoxic CD8⁺ T cells (1). After prolonged fasting or fasting-mimicking diet (FMD), an enhanced hematopoietic regeneration rate (2) and enrichment of common lymphoid progenitor cells (CLP) can occur (3). The *in vitro* adoptive T cells immunotherapy (ACI) (4) and *in vivo* nutrient changes (5) reset CD8⁺ TIL metabolism toward mitochondrial oxidative pathways, thus limiting substrate competition with cancer cells and enhancing CD8⁺ TIL-mediated immunosurveillance. Dietary strategies promoting functional gut microbiota changes (e.g., *Akkermansia muciniphila* enrichment) (6) might improve the immune-checkpoint inhibitors (anti PD1/PD-L1) efficacy (7).

of proliferating cancer cells create an immunosuppressive microenvironment (106). Indeed, the glucose-dependent CD8⁺ TIL could undergo a competitive disadvantage for nutrients, and this would negatively affect their immune function. The immunosuppressive metabolic environment could be further enhanced by tumor expression of inhibitory ligands for programmed death 1 receptor (PD-1) which, when bound to their cognate receptors on T cells, limits T cell-intrinsic glucose uptake and glycolysis (107, 108). It has been reported that KD significantly reduces the expression of the inhibitory ligand PD-1 (PD-L1) on CD8⁺ TIL (109). Additionally, mice fed with KD have reduced expression of PD-L1 on the cancer cells that notoriously inhibits CD8⁺ T cells activity (109). This suggests that KD may alter tumor-mediated T cell suppression by reducing the number of cells that are susceptible to inhibition through the PD-1 inhibitory pathway (**Figure 2**).

Nowadays, there has been intense interest in developing adoptive T cells immunotherapy (ACI), which consists in reintroducing into a patient T cells that are previously activated and expanded *in vitro* (110, 111). The success of the ACI depends on the replicative capacity of implanted T cells. A large amount of research has been directed in optimizing T cell activation and using appropriate adjuvants for ACI. However, few experimental studies have been focused on manipulating metabolic pathways that could potentially enhance immunotherapy efficacy. When posed in culture, T cells dispose of a high glucose availability, which is far from the glucose physiological levels especially in the tumor environment (112, 113). Thus, once reintroduced in patients, T cells suffer from low glucose levels and show a moderate survival and replicative capacity. It has been reported that limiting glycolysis in cultured T cells can increase their longevity without inhibiting proliferative capacity (114, 115) (**Figure 2**). Another potential way to enhance the replicative capacity and longevity of ACI cells is promoting oxidative phosphorylation and mitochondrial biogenesis *via* the inhibition of glucose-related signaling pathway that ultimately leads to *in vivo* persistence and improved antitumor immunity (116). The metabolic reprogramming of infiltrating glycolytic lymphocytes toward a catabolic state reliant on fatty acid oxidation appears to assure the success of immunotherapy (113). In line with this assumption, it was

recently demonstrated that the enhancement of lipid catabolism in CD8+ T cells increases the efficacy of immunotherapy within a tumor microenvironment low in glucose (117). In a mouse model of malignant glioma, an enhanced cytolysis *via* tumor-reactive CD8+ T cells was also achieved by ketogenic diet (109). The immunometabolic reprogramming necessary for CD8+ TIL could at least partially explain the mechanism by which KD or fasting enhances cytotoxic effect against cancer cells. Such diets are indeed powerful in inducing a cellular metabolic shift from glycolysis toward FAO.

It is now emerging that CD8+ TIL response to immune checkpoint blockade inhibitor PD1 can be also modulated by gut microbiota (118–120). A very recent paper has revealed that fecal microbiota from patients affected with metastatic melanoma and responsive to anti-PD1 therapy display increased abundance of *Akkermansia muciniphila*. *A. muciniphila* introduction into mice receiving human nonresponder fecal microbiota transplant improved antitumor immune CD8+ T cell infiltration and activity and increased anti-PD1 therapy efficacy (120, 121). Another intriguing observation is that *Faecalibacterium* and *Bifidobacterium* are associated with anti-inflammatory responses, a regulatory arm of the immune system that aims to prevent over-activation of the immune response and restores host homeostasis (120). Given that changes in host metabolism and microbiota can occur in tandem, it was hypothesized that gut microbial diversity and composition are predictors of the response to

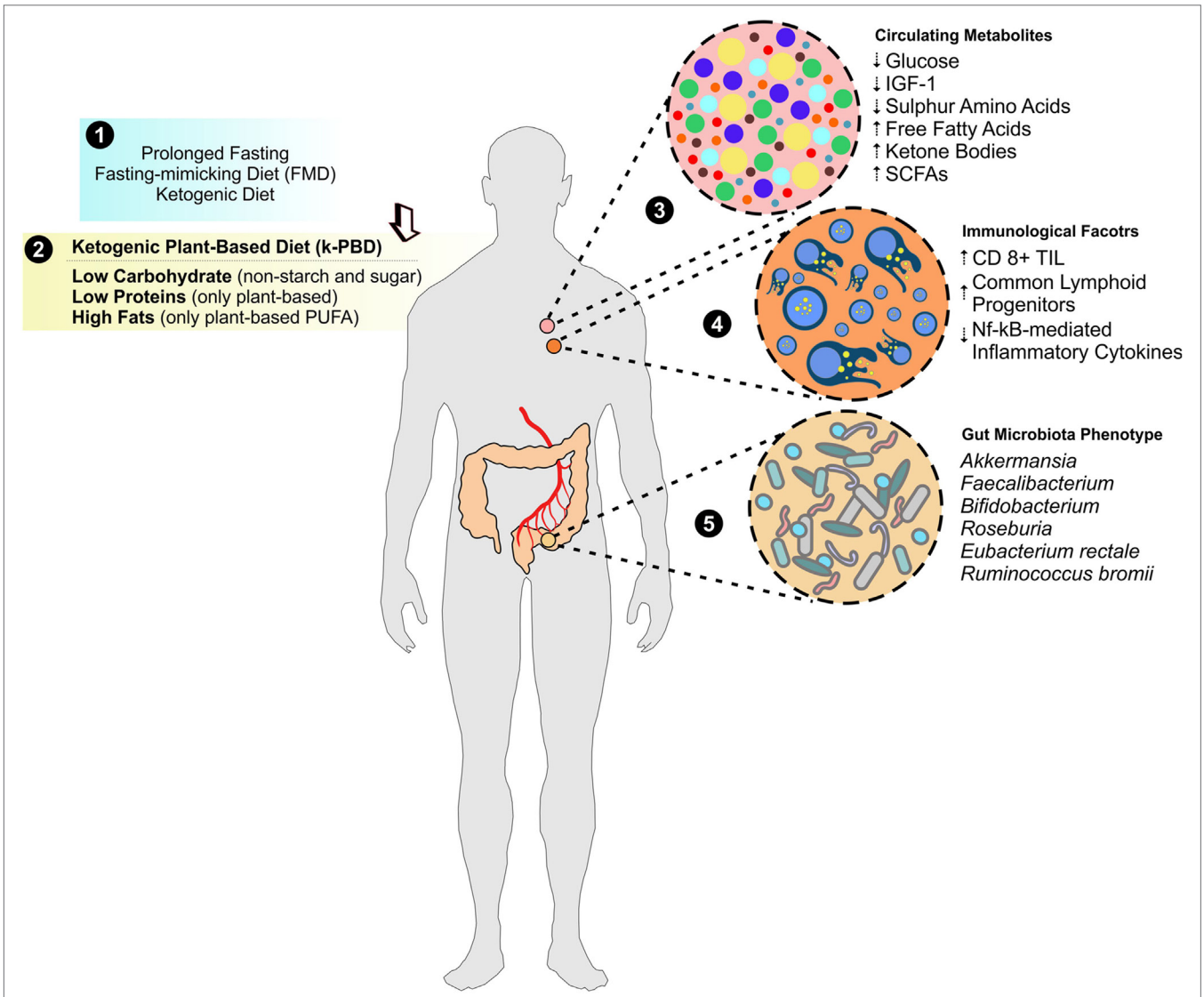


FIGURE 3 | Improving metabolites and immunological anticancer profile by k-PBD. Evidence from prolonged fasting, fasting-mimicking diet (FMD), and ketogenic diet demonstrated a strong usefulness as adjuvants in cancer therapy (1). In this issue, we propose a moderate ketogenic plant-based diet (k-PBD), low in carbohydrates (starch and sugars in particular) and animal proteins (poor in sulfur amino acids and selenium) but rich in fats [mainly in vegetable polyunsaturated fatty acids (PUFA)] (2), which could strongly modulate circulating metabolites (3), immunological factors (4), and gut microbiota asset (5) that overall create a hostile environment to cancer cells.

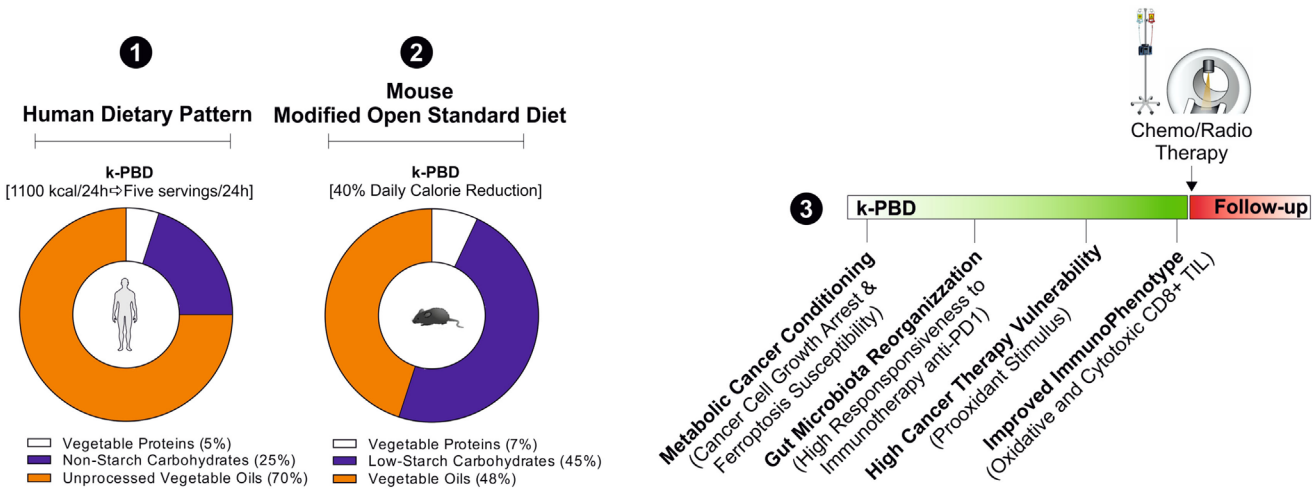


FIGURE 4 | Hypothetical cancer phenotypic responses to k-PBD. For the human dietary intervention studies, k-PBD (for 4 consecutive days; 5 servings each day) should provide about 1,100 kcal/die with 5% of calories from vegetable proteins, 25% from carbohydrates (non-starchy vegetables), and 70% from unprocessed vegetable oils (1). k-PBD proposed for murine cancer models should be reduced in total calories (40% reduction versus *ad libitum* diet), provide 7% calories from vegetable proteins, 45% from low starch carbohydrates, 48% in vegetable oils (2). The proposed dietary pattern should be started prior to conventional cancer therapies (3).

cancer therapy (121) (**Figure 2**). Accordingly, germ-free mice implanted with human tumor cells and transplanted with feces from chemotherapy responders showed an ameliorated response to chemotherapy than mice colonized with microbiota from nonresponder patients (119).

The diet has a strong capacity to rapidly and reproducibly reshape the gut microbiome (122). Indeed, fasting or plant-based diet remodels microbial community structure and overwhelms interindividual differences in microbial gene expression. The animal-based diets are known to increase the abundance of bile-tolerant microorganisms (*Alistipes*, *Bilophila*, and *Bacteroides*) and decrease the levels of the high fermentative *Firmicutes* that metabolize dietary plant polysaccharides (*Roseburia, Eubacterium rectale*, and *Ruminococcus bromii*) (122). More recently, it has been demonstrated that alternate day fasting shifts the gut microbiota composition from *Bacteroides* to *Firmicutes* leading to elevation of the fermentation products (123). Plant-based foods are mainly characterized by resistant starches and dietary fibers and promote their gut microbiota-mediated fermentation and decomposition. These processes provide additional amount of short chain fatty acids (SCFAs) to the host (124) (**Figure 2**). The major SCFAs, i.e., acetate, propionate, and butyrate, have different production ratios and physiological activities. Through ^{1}H NMR-based metabolomics, it was revealed that mice treated with alternate day fasting increased acetate levels both in the cecum and sera (123). Acetate, when ligated to coenzyme A (acetyl-CoA), is among the most central and dynamic metabolites in intermediary metabolism. Under stressful circumstance (e.g., fasting-like conditions), cancer cells may convert extracellular acetate to acetyl-CoA, thus promoting the biogenesis of membrane building blocks that sustain the high proliferative rate. This adaptive response involves the cytosolic form of short-chain acyl-CoA synthetases (ACC2). Accordingly, increased ACC2 protein levels were detected in a subset of human triple negative breast cancer samples, and such an elevation correlates with poor survival (125). Differently to acetate, butyrate shows many regulatory properties including the inhibition of histone deacetylases. Histone deacetylase inhibitors (HDACi's) are emerging as promising anticancer drugs when administered alone or in combination with chemotherapeutic agents or radiotherapy. Previous research suggests that HDACi's have a high degree of selectivity for killing cancer cells. For instance, the HDACi sodium butyrate suppresses DNA double strand break repair induced by etoposide more efficiently in MCF-7 cells than in HEK293 cells (126). Sodium butyrate alone also resulted in accumulation of ROS, DNA double-strand breaks, and apoptosis in HCT-116 colon cancer cell lines; when combined with mitomycin C or radiotherapy, sodium butyrate increases sensitivity of cancer cells to the drug (127, 128). In animal models of gastric carcinoma, sodium butyrate was found to inhibit tumor mass formation and increase tumor infiltration by $CD8^{+}$ TIL (129). Finally, several studies also demonstrated a strong effectiveness of SCFA to inactivate nuclear factor-kb by downregulating the production of the pro-inflammatory cytokine TNFα (130–134), which is commonly activated to promote a pro-carcinogenic environmental milieu (135) (**Figure 1**).

CONCLUSION AND PERSPECTIVE

Despite recent advances have been made in cancer therapy, the prognosis for many cancer patients remains poor, and current treatments still show severe adverse events. Thus, finding complementary treatments that have limited patient toxicity and simultaneously enhance therapy responses in cancer versus normal cells is urgent. Diet has a strong capacity to modulate cell responses to environmental stimuli and shows great potential in

improving cancer prognosis. The mechanisms by which dietary nutrients enhance anticancer effects of standard anticancer therapies (chemotherapy, radiotherapy, immunotherapy) has not been fully elucidated yet. Preclinical studies have demonstrated the safety and efficacy of specific dietary interventions in counteracting tumor progression during anticancer therapy in murine models. However, most of the data present in the literature take advantage of the use of mice and this may limit the translation to clinical research. Therefore, a huge amount of work is now necessary to confirm these very promising results in humans.

Deprivation of nutrients (e.g., glucose, sulfur amino acids) as well as of nutrient-responsive growth factors (e.g., IGF-1) seems to selectively kill high proliferative/resilient cancer cells by forcing their glycolytic asset toward an oxidative metabolism (i.e., fatty acids and ketone bodies as energy sources) and limiting GPX activity as consequence of reduced GSH levels. Nutrient scarcity also improves immunometabolism enhancing cytotoxic efficiency of $CD8^+$ TIL within the tumor mass through, probably, the concomitant gut microbiota and immunometabolic rearrangements (**Figure 3**).

Herein, we propose weekly cycles of 4 days of a plant-based moderate ketogenic diet (k-PBD) that could reprogram systemic metabolism conferring a hostile environment to cancer cells (**Figure 3**). In particular, k-PBD should be low in proteins (mainly vegetable proteins low in sulfur amino acids and selenium), carbohydrates (non-starchy vegetables), and high in lipids (mainly unprocessed vegetable oils rich in PUFA). Remarkably, even though not supported by experimental data, it is highly expected that this diet could be able to increase ketonemia as it contains high amounts of fats concomitantly to reduced calories. This diet could increase the efficiency of $CD8^+$ TIL, by reprogramming their metabolism (fat-dependent metabolism) to better counteract the metabolic features of proliferating cancer cells (glucose-dependent metabolism) and sensitize cancer cells to the therapy. The k-PBD could be consumed prior to conventional cancer therapies (e.g., prior each cycle of chemotherapy or prior a single fraction of radiation therapy). With this composition and time of treatment, k-PBD could be effective in: (i) changing the membrane chemistry by PUFA enrichment (high peroxidation index); (ii) reducing the sulfur-dependent antioxidant power (lowering NADPH, GSH, GPX4); (iii) forcing the metabolic shift toward mitochondrial metabolism in cancer cells. Furthermore, the high fermentative fibers of k-PBD could induce a functional microbiota reshaping improving immunotherapy efficacy (e.g., anti-PD1 therapy) (**Figure 4**).

AUTHOR CONTRIBUTIONS

DL-B conceptualized and wrote the manuscript. KA performed critical revision of the manuscript for intellectual content.

REFERENCES

1. Berrigan D, Perkins SN, Haines DC, Hursting SD. Adult-onset calorie restriction and fasting delay spontaneous tumorigenesis in p53-deficient mice. *Carcinogenesis* (2002) 23:817–22. doi:10.1093/carcin/23.5.817
2. Levine ME, Suarez JA, Brandhorst S, Balasubramanian P, Cheng CW, Madia F, et al. Low protein intake is associated with a major reduction in IGF-1, cancer, and overall mortality in the 65 and younger but not older population. *Cell Metab* (2014) 19:407–17. doi:10.1016/j.cmet.2014.02.006
3. Cavuoto P, Fenech MF. A review of methionine dependency and the role of methionine restriction in cancer growth control and life-span extension. *Cancer Treat Rev* (2012) 38:726–36. doi:10.1016/j.ctrv.2012.01.004
4. Fontana L, Partridge L, Longo VD. Extending healthy life span – from yeast to humans. *Science* (2010) 328:321–6. doi:10.1126/science.1172539
5. Kritchevsky D. Caloric restriction and cancer. *J Nutr Sci Vitaminol (Tokyo)* (2001) 47:13–9. doi:10.3177/jnsv.47.13
6. Nogueira LM, Dunlap SM, Ford NA, Hursting SD. Calorie restriction and rapamycin inhibit MMTV-Wnt-1 mammary tumor growth in a mouse model of postmenopausal obesity. *Endocr Relat Cancer* (2012) 19:57–68. doi:10.1530/ERC-11-0213
7. Saleh AD, Simone BA, Palazzo J, Savage JE, Sano Y, Dan T, et al. Caloric restriction augments radiation efficacy in breast cancer. *Cell Cycle* (2013) 12:1955–63. doi:10.4161/cc.25016
8. Brandhorst S, Longo VD. Fasting and caloric restriction in cancer prevention and treatment. *Recent Results Cancer Res* (2016) 207:241–66. doi:10.1007/978-3-319-42118-6_12
9. Lettieri-Barbato D, Giovannetti E, Aquilano K. Effects of dietary restriction on adipose mass and biomarkers of healthy aging in human. *Aging (Albany NY)* (2016) 8:3341–55. doi:10.18632/aging.101122
10. Lettieri Barbato D, Aquilano K. Feast and famine: adipose tissue adaptations for healthy aging. *Ageing Res Rev* (2016) 28:85–93. doi:10.1016/j.arr.2016.05.007
11. Lettieri Barbato D, Tatulli G, Aquilano K, Ciriolo MR, et al. Mitochondrial hormesis links nutrient restriction to improved metabolism in fat cell. *Aging (Albany NY)* (2015) 7:869–81. doi:10.18632/aging.100832
12. Zarse K, Schmeisser S, Groth M, Priebe S, Beuster G, Kuhlow D, et al. Impaired insulin/IGF1 signaling extends life span by promoting mitochondrial L-proline catabolism to induce a transient ROS signal. *Cell Metab* (2012) 15:451–65. doi:10.1016/j.cmet.2012.02.013
13. Longo VD. Linking sirtuins, IGF-I signaling, and starvation. *Exp Gerontol* (2009) 44:70–4. doi:10.1016/j.exger.2008.06.005
14. Safdie F, Brandhorst S, Wei M, Wang W, Lee C, Hwang S, et al. Fasting enhances the response of glioma to chemo- and radiotherapy. *PLoS One* (2012) 7:e44603. doi:10.1371/journal.pone.0044603
15. Lee C, Raffaghello L, Brandhorst S, Safdie FM, Bianchi G, Martin-Montalvo A, et al. Fasting cycles retard growth of tumors and sensitize a range of cancer cell types to chemotherapy. *Sci Transl Med* (2012) 4:124ra127. doi:10.1126/scitranslmed.3003293
16. Lee C, Safdie FM, Raffaghello L, Wei M, Madia F, Parrella E, et al. Reduced levels of IGF-I mediate differential protection of normal and cancer cells in response to fasting and improve chemotherapeutic index. *Cancer Res* (2010) 70:1564–72. doi:10.1158/0008-5472.CAN-09-3228
17. Di Biase S, Lee C, Brandhorst S, Manes B, Buono R, Cheng CW, et al. Fasting-mimicking diet reduces HO-1 to promote T cell-mediated tumor cytotoxicity. *Cancer Cell* (2016) 30:136–46. doi:10.1016/j.ccell.2016.06.005
18. Di Biase S, Longo VD. Fasting-induced differential stress sensitization in cancer treatment. *Mol Cell Oncol* (2016) 3:e1117701. doi:10.1080/23723556.2015.1117701
19. Di Biase S, Shim HS, Kim KH, Vinciguerra M, Rappa F, Wei M, et al. Fasting regulates EGR1 and protects from glucose- and dexamethasone-dependent sensitization to chemotherapy. *PLoS Biol* (2017) 15:e2001951. doi:10.1371/journal.pbio.2001951
20. Raffaghello L, Lee C, Safdie FM, Wei M, Madia F, Bianchi G, et al. Starvation-dependent differential stress resistance protects normal but not cancer cells against high-dose chemotherapy. *Proc Natl Acad Sci U S A* (2008) 105:8215–20. doi:10.1073/pnas.0708100105
21. Longo VD, Panda S. Fasting, circadian rhythms, and time-restricted feeding in healthy lifespan. *Cell Metab* (2016) 23:1048–59. doi:10.1016/j.cmet.2016.06.001
22. Longo VD, Mattson MP. Fasting: molecular mechanisms and clinical applications. *Cell Metab* (2014) 19:181–92. doi:10.1016/j.cmet.2013.12.008
23. Klement RJ, Champ CE, Otto C, Kämmerer U. Anti-tumor effects of ketogenic diets in mice: a meta-analysis. *PLoS One* (2016) 11:e0155050. doi:10.1371/journal.pone.0155050

24. Allen BG, Bhatia SK, Anderson CM, Eichenberger-Gilmore JM, Sibenaller ZA, Mapuskar KA, et al. Ketogenic diets as an adjuvant cancer therapy: history and potential mechanism. *Redox Biol* (2014) 2:963–70. doi:10.1016/j.redox.2014.08.002
25. Zahra A, Fath MA, Opat E, Mapuskar KA, Bhatia SK, Ma DC, et al. Consuming a ketogenic diet while receiving radiation and chemotherapy for locally advanced lung cancer and pancreatic cancer: the university of iowa experience of two phase 1 clinical trials. *Radiat Res* (2017) 187:743–54. doi:10.1667/RR14668.1
26. Allen BG, Bhatia SK, Buatti JM, Brandt KE, Lindholm KE, Button AM, et al. Ketogenic diets enhance oxidative stress and radio-chemo-therapy responses in lung cancer xenografts. *Clin Cancer Res* (2013) 19:3905–13. doi:10.1158/1078-0432.CCR-12-0287
27. Zhou PT, Li B, Liu FR, Zhang MC, Wang Q, Li YY, et al. Metformin is associated with survival benefit in pancreatic cancer patients with diabetes: a systematic review and meta-analysis. *Oncotarget* (2017) 8:25242–50. doi:10.18632/oncotarget.15692
28. Jung YS, Park CH, Eun CS, Park DI, Han DS. Metformin use and the risk of colorectal adenoma: a systematic review and meta-analysis. *J Gastroenterol Hepatol* (2017) 32:957–65. doi:10.1111/jgh.13639
29. Pernicova I, Korbonits M. Metformin – mode of action and clinical implications for diabetes and cancer. *Nat Rev Endocrinol* (2014) 10:143–56. doi:10.1038/nrendo.2013.256
30. Samsuri NAB, Leech M, Marignol L. Metformin and improved treatment outcomes in radiation therapy – a review. *Cancer Treat Rev* (2017) 55:150–62. doi:10.1016/j.ctrv.2017.03.005
31. Pavel ME, Singh S, Strosberg JR, Bubuteishvili-Pacaud L, Degtyarev E, Neary MP, et al. Health-related quality of life for everolimus versus placebo in patients with advanced, non-functional, well-differentiated gastrointestinal or lung neuroendocrine tumours (RADIANT-4): a multicentre, randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol* (2017) 18:1411–22. doi:10.1016/S1470-2045(17)30471-0
32. Kasprzak A, Kwasniewski W, Adamek A, Gozdzicka-Jozefiak A. Insulin-like growth factor (IGF) axis in cancerogenesis. *Mutat Res Rev Mutat Res* (2017) 772:78–104. doi:10.1016/j.mrrev.2016.08.007
33. Desideri E, Vegliante R, Cardaci S, Nepravishta R, Paci M, Ciriolo MR, et al. MAPK14/p38alpha-dependent modulation of glucose metabolism affects ROS levels and autophagy during starvation. *Autophagy* (2014) 10:1652–65. doi:10.4161/auto.29456
34. Poillet-Perez L, Despouy G, Delage-Mourroux R, Boyer-Guittaut M. Interplay between ROS and autophagy in cancer cells, from tumor initiation to cancer therapy. *Redox Biol* (2015) 4:184–92. doi:10.1016/j.redox.2014.12.003
35. Antunes F, Pereira GJ, Jasiulionis MG, Bincoletto C, Smaili SS. Nutritional shortage augments cisplatin-effects on murine melanoma cells. *Chem Biol Interact* (2018) 281:89–97. doi:10.1016/j.cbi.2017.12.027
36. Bianchi G, Martella R, Ravera S, Marini C, Capitanio S, Orengo A, et al. Fasting induces anti-Warburg effect that increases respiration but reduces ATP-synthesis to promote apoptosis in colon cancer models. *Oncotarget* (2015) 6:11806–19. doi:10.18632/oncotarget.3688
37. Ahmad IM, Aykin-Burns N, Sim JE, Walsh SA, Higashikubo R, Buettner GR, et al. Mitochondrial O_2*- and H2O2 mediate glucose deprivation-induced stress in human cancer cells. *J Biol Chem* (2005) 280:4254–63. doi:10.1074/jbc.M411662200
38. Hue L, Taegtmeyer H. The Randle cycle revisited: a new head for an old hat. *Am J Physiol Endocrinol Metab* (2009) 297:E578–91. doi:10.1152/ajpendo.00093.2009
39. Stanton RC. Glucose-6-phosphate dehydrogenase, NADPH, and cell survival. *IUBMB Life* (2012) 64:362–9. doi:10.1002/iub.1017
40. Moreno-Sánchez R, Gallardo-Pérez JC, Rodríguez-Enríquez S, Saavedra E, Marín-Hernández Á. Control of the NADPH supply for oxidative stress handling in cancer cells. *Free Radic Biol Med* (2017) 112:149–61. doi:10.1016/j.freeradbiomed.2017.07.018
41. Imai H, Nakagawa Y. Biological significance of phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammalian cells. *Free Radic Biol Med* (2003) 34:145–69. doi:10.1016/S0891-5849(02)01197-8
42. Jain SK, Kannan K, Lim G. Ketosis (acetoacetate) can generate oxygen radicals and cause increased lipid peroxidation and growth inhibition in human endothelial cells. *Free Radic Biol Med* (1998) 25:1083–8. doi:10.1016/S0891-5849(98)00140-3
43. Aquilano K, Baldelli S, Ciriolo MR. Glutathione: new roles in redox signaling for an old antioxidant. *Front Pharmacol* (2014) 5:196. doi:10.3389/fphar.2014.00196
44. Ciccarese F, Ciminale V. Escaping death: mitochondrial redox homeostasis in cancer cells. *Front Oncol* (2017) 7:117. doi:10.3389/fonc.2017.00117
45. Qu Q, Zeng F, Liu X, et al. Fatty acid oxidation and carnitine palmitoyltransferase I: emerging therapeutic targets in cancer. *Cell Death Dis* (2016) 7:e2226. doi:10.1038/cddis.2016.132
46. Patra KC, Hay N. The pentose phosphate pathway and cancer. *Trends Biochem Sci* (2014) 39:347–54. doi:10.1016/j.tibs.2014.06.005
47. Lettieri Barbato D, Vegliante R, Desideri E, Ciriolo MR. Managing lipid metabolism in proliferating cells: new perspective for metformin usage in cancer therapy. *Biochim Biophys Acta* (2014) 1845:317–24. doi:10.1016/j.bbcan.2014.02.003
48. Carracedo A, Cantley LC, Pandolfi PP. Cancer metabolism: fatty acid oxidation in the limelight. *Nat Rev Cancer* (2013) 13:227–32. doi:10.1038/nrc3483
49. Rohrig F, Schulze A. The multifaceted roles of fatty acid synthesis in cancer. *Nat Rev Cancer* (2016) 16:732–49. doi:10.1038/nrc.2016.89
50. Yao CH, Fowle-Grider R, Mahieu NG, Liu GY, Chen YJ, Wang R, et al. Exogenous fatty acids are the preferred source of membrane lipids in proliferating fibroblasts. *Cell Chem Biol* (2016) 23:483–93. doi:10.1016/j.chembiol.2016.03.007
51. Egnatchik RA, DeBerardinis RJ. Liposuction: extracellular fat removal promotes proliferation. *Cell Chem Biol* (2016) 23:431–2. doi:10.1016/j.chembiol.2016.04.003
52. Berquin IM, Edwards IJ, Chen YQ. Multi-targeted therapy of cancer by omega-3 fatty acids. *Cancer Lett* (2008) 269:363–77. doi:10.1016/j.canlet.2008.03.044
53. Serini S, Piccioni E, Merendino N, Calviello G. Dietary polyunsaturated fatty acids as inducers of apoptosis: implications for cancer. *Apoptosis* (2009) 14:135–52. doi:10.1007/s10495-008-0298-2
54. Vaughan VC, Hassing MR, Lewandowski PA. Marine polyunsaturated fatty acids and cancer therapy. *Br J Cancer* (2013) 108:486–92. doi:10.1038/bjc.2012.586
55. Biondo PD, Brindley DN, Sawyer MB, Field CJ. The potential for treatment with dietary long-chain polyunsaturated n-3 fatty acids during chemotherapy. *J Nutr Biochem* (2008) 19:787–96. doi:10.1016/j.jnutbio.2008.02.003
56. D'Eliseo D, Velotti F. Omega-3 fatty acids and cancer cell cytotoxicity: implications for multi-targeted cancer therapy. *J Clin Med* (2016) 5:ii:E15. doi:10.3390/jcm5020015
57. Jenkinson A, Franklin MF, Wahle K, Duthie GG. Dietary intakes of polyunsaturated fatty acids and indices of oxidative stress in human volunteers. *Eur J Clin Nutr* (1999) 53:523–8. doi:10.1038/sj.ejcn.1600783
58. Diniz YS, Cicogna AC, Padovani CR, Santana LS, Faine LA, Novelli EL. Diets rich in saturated and polyunsaturated fatty acids: metabolic shifting and cardiac health. *Nutrition* (2004) 20:230–4. doi:10.1016/j.nut.2003.10.012
59. Agmon E, Stockwell BR. Lipid homeostasis and regulated cell death. *Curr Opin Chem Biol* (2017) 39:83–9. doi:10.1016/j.cbpa.2017.06.002
60. Barelli H, Antonny B. Lipid unsaturation and organelle dynamics. *Curr Opin Cell Biol* (2016) 41:25–32. doi:10.1016/j.ceb.2016.03.012
61. Ferretti A, Chen LL, Di Vito M, Barca S, Tombesi M, Cianfriglia M, et al. Pentose phosphate pathway alterations in multi-drug resistant leukemic T-cells: 31P NMR and enzymatic studies. *Anticancer Res* (1993) 13:867–72.
62. Friesen C, Kiess Y, Debatin KM. A critical role of glutathione in determining apoptosis sensitivity and resistance in leukemia cells. *Cell Death Differ* (2004) 11(Suppl 1):S73–85. doi:10.1038/sj.cdd.4401431
63. Gessner T, Vaughan LA, Beehler BC, Bartels CJ, Baker RM. Elevated pentose cycle and glucuronyltransferase in daunorubicin-resistant P388 cells. *Cancer Res* (1990) 50:3921–7.
64. Chen JJ, Galluzzi L. Fighting resilient cancers with iron. *Trends Cell Biol* (2018) 28:77–8. doi:10.1016/j.tcb.2017.11.007
65. Viswanathan VS, Ryan MJ, Dhruv HD, Gill S, Eichhoff OM, Seashore-Ludlow B, et al. Dependency of a therapy-resistant state of cancer cells on a lipid peroxidase pathway. *Nature* (2017) 547:453–7. doi:10.1038/nature23007
66. Burk RF. Protection against free radical injury by selenoenzymes. *Pharmacol Ther* (1990) 45:383–5. doi:10.1016/0163-7258(90)90073-B
67. Girotti AW. Lipid hydroperoxide generation, turnover, and effector action in biological systems. *J Lipid Res* (1998) 39:1529–42.

68. Thomas JP, Maiorino M, Ursini F, Girotti AW. Protective action of phospholipid hydroperoxide glutathione peroxidase against membrane-damaging lipid peroxidation. In situ reduction of phospholipid and cholesterol hydroperoxides. *J Biol Chem* (1990) 265:454–61.
69. Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, et al. Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell* (2012) 149:1060–72. doi:10.1016/j.cell.2012.03.042
70. Ingold I, Berndt C, Schmitt S, Doll S, Poschmann G, Buday K, et al. Selenium utilization by GPX4 is required to prevent hydroperoxide-induced ferroptosis. *Cell* (2018) 172:409–422.e21. doi:10.1016/j.cell.2017.11.048
71. Stockwell BR, Friedmann Angeli JP, Bayir H, Bush AI, Conrad M, Dixon SJ, et al. Ferroptosis: a regulated cell death nexus linking metabolism, redox biology, and disease. *Cell* (2017) 171:273–85. doi:10.1016/j.cell.2017.09.021
72. Dixon SJ, Patel DN, Welsch M, Skouta R, Lee ED, Hayano M, et al. Pharmacological inhibition of cystine-glutamate exchange induces endoplasmic reticulum stress and ferroptosis. *Elife* (2014) 3:e02523. doi:10.7554/eLife.02523
73. Toyokuni S, Ito F, Yamashita K, Okazaki Y, Akatsuka S. Iron and thiol redox signaling in cancer: an exquisite balance to escape ferroptosis. *Free Radic Biol Med* (2017) 108:610–26. doi:10.1016/j.freeradbiomed.2017.04.024
74. Hellmich MR, Coletta C, Chao C, Szabo C, et al. The therapeutic potential of cystathionine beta-synthetase/hydrogen sulfide inhibition in cancer. *Antioxid Redox Signal* (2015) 22:424–48. doi:10.1089/ars.2014.5933
75. Cramer SL, Saha A, Liu J, Tadi S, Tiziani S, Yan W, et al. Systemic depletion of L-cyst(e)ine with cyst(e)inase increases reactive oxygen species and suppresses tumor growth. *Nat Med* (2017) 23:120–7. doi:10.1038/nm.4232
76. Liu DS, Duong CP, Haupt S, Montgomery KG, House CM, Azar WJ, et al. Inhibiting the system xC(-)/glutathione axis selectively targets cancers with mutant-p53 accumulation. *Nat Commun* (2017) 8:14844. doi:10.1038/ncomms14844
77. Louandre C, Ezzoukhry Z, Godin C, Barbare JC, Mazière JC, Chauffert B, et al. Iron-dependent cell death of hepatocellular carcinoma cells exposed to sorafenib. *Int J Cancer* (2013) 133:1732–42. doi:10.1002/ijc.28159
78. Desideri E, Filomeni G, Ciriolo MR. Glutathione participates in the modulation of starvation-induced autophagy in carcinoma cells. *Autophagy* (2012) 8:1769–81. doi:10.4161/auto.22037
79. Giustarini D, Dalle-Donne I, Milzani A, Rossi R. Oxidative stress induces a reversible flux of cysteine from tissues to blood in vivo in the rat. *FEBS J* (2009) 276:4946–58. doi:10.1111/j.1742-4658.2009.07197.x
80. Yu X, Long YC. Crosstalk between cystine and glutathione is critical for the regulation of amino acid signaling pathways and ferroptosis. *Sci Rep* (2016) 6:30033. doi:10.1038/srep30033
81. Stipanuk MH. Metabolism of sulfur-containing amino acids. *Annu Rev Nutr* (1986) 6:179–209. doi:10.1146/annurev.nu.06.070186.001143
82. Finkelstein JD, Martin JJ, Harris BJ. Methionine metabolism in mammals. The methionine-sparing effect of cystine. *J Biol Chem* (1988) 263:11750–4.
83. Lien EC, Ghisolfi L, Geck RC, Asara JM, Toker A. Oncogenic PI3K promotes methionine dependency in breast cancer cells through the cystine-glutamate antiporter xCT. *Sci Signal* (2017) 10:eaao6604. doi:10.1126/scisignal.aao6604
84. Strekalova E, Malin D, Good DM, Cryns VL. Methionine deprivation induces a targetable vulnerability in triple-negative breast cancer cells by enhancing TRAIL receptor-2 expression. *Clin Cancer Res* (2015) 21:2780–91. doi:10.1158/1078-0432.CCR-14-2792
85. Hoffman RM. Development of recombinant methioninase to target the general cancer-specific metabolic defect of methionine dependence: a 40-year odyssey. *Expert Opin Biol Ther* (2015) 15:21–31. doi:10.1517/14712598.2015.963050
86. Hoffman RM. Methionine dependence in cancer cells – a review. *In Vitro* (1982) 18:421–8. doi:10.1007/BF02796353
87. Cao WX, Ou JM, Fei XF, Zhu ZG, Yin HR, Yan M, et al. Methionine-dependence and combination chemotherapy on human gastric cancer cells in vitro. *World J Gastroenterol* (2002) 8:230–2. doi:10.3748/wjg.v8.i2.230
88. Finkelstein JD. Methionine metabolism in mammals. *J Nutr Biochem* (1990) 1:228–37. doi:10.1016/0955-2863(90)90070-2
89. Finkelstein JD, Kyle WE, Harris BJ. Methionine metabolism in mammals: regulatory effects of S-adenosylhomocysteine. *Arch Biochem Biophys* (1974) 165:774–9. doi:10.1016/0003-9861(74)90306-3
90. Lo M, Ling V, Wang YZ, Gout PW. The xc- cystine/glutamate antiporter: a mediator of pancreatic cancer growth with a role in drug resistance. *Br J Cancer* (2008) 99:464–72. doi:10.1038/sj.bjc.6604485
91. Doxsee DW, Gout PW, Kurita T, Lo M, Buckley AR, Wang Y, et al. Sulfasalazine-induced cystine starvation: potential use for prostate cancer therapy. *Prostate* (2007) 67:162–71. doi:10.1002/pros.20508
92. Rennekamp AJ. The ferrous awakens. *Cell* (2017) 171:1225–7. doi:10.1016/j.cell.2017.11.029
93. Szabo C, Coletta C, Chao C, Módis K, Szczesny B, Papapetropoulos A, et al. Tumor-derived hydrogen sulfide, produced by cystathionine-beta-synthase, stimulates bioenergetics, cell proliferation, and angiogenesis in colon cancer. *Proc Natl Acad Sci U S A* (2013) 110:12474–9. doi:10.1073/pnas.1306241110
94. Cardaci S, Rizza S, Filomeni G, Bernardini R, Bertocchi F, Mattei M, et al. Glutamine deprivation enhances antitumor activity of 3-bromopyruvate through the stabilization of monocarboxylate transporter-1. *Cancer Res* (2012) 72:4526–36. doi:10.1158/0008-5472.CAN-12-1741
95. Vegliante R, Desideri E, Di Leo L, Ciriolo MR. Dehydroepiandrosterone triggers autophagic cell death in human hepatoma cell line HepG2 via JNK-mediated p62/SQSTM1 expression. *Carcinogenesis* (2016) 37:233–44. doi:10.1093/carcin/bgw003
96. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* (2016) 12:1–222. doi:10.1080/15548627.2015.1100356
97. Gao M, Monian P, Pan Q, Zhang W, Xiang J, Jiang X. Ferroptosis is an autophagic cell death process. *Cell Res* (2016) 26:1021–32. doi:10.1038/cr.2016.95
98. Hou W, Xie Y, Song X, Sun X, Lotze MT, Zeh HJ III, et al. Autophagy promotes ferroptosis by degradation of ferritin. *Autophagy* (2016) 12:1425–8. doi:10.1080/15548627.2016.1187366
99. Ma S, Dielschneider RF, Henson ES, Xiao W, Choquette TR, Blankstein AR, et al. Ferroptosis and autophagy induced cell death occur independently after siramesine and lapatinib treatment in breast cancer cells. *PLoS One* (2017) 12:e0182921. doi:10.1371/journal.pone.0182921
100. Pietrocola F, Pol J, Vacchelli E, Rao S, Enot DP, Baracco EE, et al. Caloric restriction mimetics enhance anticancer immunosurveillance. *Cancer Cell* (2016) 30:147–60. doi:10.1016/j.ccell.2016.05.016
101. Rubio-Patino C, Bossowski JP, De Donatis GM, Mondragón L, Villa E, Aira LE, et al. Low-protein diet induces IRE1alpha-dependent anticancer immunosurveillance. *Cell Metab* (2018) 27(4):828–42.e7. doi:10.1016/j.cmet.2018.02.009
102. Cheng CW, Adams GB, Perin L, Wei M, Zhou X, Lam BS, et al. Prolonged fasting reduces IGF-1/PKA to promote hematopoietic-stem-cell-based regeneration and reverse immunosuppression. *Cell Stem Cell* (2014) 14:810–23. doi:10.1016/j.stem.2014.04.014
103. Brandhorst S, Choi IY, Wei M, Cheng CW, Sedrakyan S, Navarrete G, et al. A periodic diet that mimics fasting promotes multi-system regeneration, enhanced cognitive performance, and healthspan. *Cell Metab* (2015) 22:86–99. doi:10.1016/j.cmet.2015.05.012
104. Andre F, Dieci MV, Dubsky P, Sotiriou C, Curigliano G, Denkert C, et al. Molecular pathways: involvement of immune pathways in the therapeutic response and outcome in breast cancer. *Clin Cancer Res* (2013) 19:28–33. doi:10.1158/1078-0432.CCR-11-2701
105. Issa-Nummer Y, Darb-Esfahani S, Loibl S, Kunz G, Nekljudova V, Schrader I, et al. Prospective validation of immunological infiltrate for prediction of response to neoadjuvant chemotherapy in HER2-negative breast cancer – a substudy of the Neoadjuvant GeparQuinto Trial. *PLoS One* (2013) 8:e79775. doi:10.1371/journal.pone.0079775
106. Pearce EL, Pearce EJ. Metabolic pathways in immune cell activation and quiescence. *Immunity* (2013) 38:633–43. doi:10.1016/j.immuni.2013.04.005
107. Ahmadzadeh M, Johnson LA, Heemskerk B, Wunderlich JR, Dudley ME, White DE, et al. Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood* (2009) 114:1537–44. doi:10.1182/blood-2008-12-195792
108. Parry RV, Chemnitz JM, Frauwirth KA, Lanfranco AR, Braunstein I, Kobayashi SV, et al. CTLA-4 and PD-1 receptors inhibit T-cell activation

by distinct mechanisms. *Mol Cell Biol* (2005) 25:9543–53. doi:10.1128/MCB.25.21.9543-9553.2005
109. Lussier DM, Woolf EC, Johnson JL, Brooks KS, Blattman JN, Scheck AC. Enhanced immunity in a mouse model of malignant glioma is mediated by a therapeutic ketogenic diet. *BMC Cancer* (2016) 16:310. doi:10.1186/s12885-016-2337-7
110. Kalos M, June CH. Adoptive T cell transfer for cancer immunotherapy in the era of synthetic biology. *Immunity* (2013) 39:49–60. doi:10.1016/j.immuni.2013.07.002
111. Bonini C, Mondino A. Adoptive T-cell therapy for cancer: the era of engineered T cells. *Eur J Immunol* (2015) 45:2457–69. doi:10.1002/eji.201545552
112. Marelli-Berg FM, Fu H, Mauro C. Molecular mechanisms of metabolic reprogramming in proliferating cells: implications for T-cell-mediated immunity. *Immunology* (2012) 136:363–9. doi:10.1111/j.1365-2567.2012.03583.x
113. Chang CH, Pearce EL. Emerging concepts of T cell metabolism as a target of immunotherapy. *Nat Immunol* (2016) 17:364–8. doi:10.1038/ni.3415
114. Pearce EL, Poffenberger MC, Chang CH, Jones RG. Fueling immunity: insights into metabolism and lymphocyte function. *Science* (2013) 342:1242454. doi:10.1126/science.1242454
115. Sukumar M, Liu J, Ji Y, Subramanian M, Crompton JG, Yu Z, et al. Inhibiting glycolytic metabolism enhances CD8+ T cell memory and antitumor function. *J Clin Invest* (2013) 123:4479–88. doi:10.1172/JCI69589
116. Crompton JG, Sukumar M, Roychoudhuri R, Clever D, Gros A, Eil RL, et al. Akt inhibition enhances expansion of potent tumor-specific lymphocytes with memory cell characteristics. *Cancer Res* (2015) 75:296–305. doi:10.1158/0008-5472.CAN-14-2277
117. Zhang Y, Kurupati R, Liu L, Zhou XY, Zhang G, Hudaihed A, et al. Enhancing CD8(+) T cell fatty acid catabolism within a metabolically challenging tumor microenvironment increases the efficacy of melanoma immunotherapy. *Cancer Cell* (2017) 32:377–391.e9. doi:10.1016/j.ccell.2017.08.004
118. Vetizou M, Trinchieri G. Anti-PD1 in the wonder-gut-land. *Cell Res* (2018) 28(3):263–4. doi:10.1038/cr.2018.12
119. Gopalakrishnan V, Spencer CN, Nezi L, Reuben A, Andrews MC, Karpinets TV, et al. Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. *Science* (2018) 359:97–103. doi:10.1126/science.aan4236
120. Matson V, Fessler J, Bao R, Chongsuwat T, Zha Y, Alegre ML, et al. The commensal microbiome is associated with anti-PD-1 efficacy in metastatic melanoma patients. *Science* (2018) 359:104–8. doi:10.1126/science.aao3290
121. Routy B, Le Chatelier E, Derosa L, Duong CPM, Alou MT, Daillère R, et al. Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. *Science* (2018) 359:91–7. doi:10.1126/science.aan3706
122. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. *Nature* (2014) 505:559–63. doi:10.1038/nature12820
123. Li GL, Xie C, Lu SY, Nichols RG, Tian Y, Li L, et al. Intermittent fasting promotes white adipose browning and decreases obesity by shaping the gut microbiota. *Cell Metab* (2017) 26:672.e–85.e. doi:10.1016/j.cmet.2017.10.007
124. Morrison DJ, Preston T. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes* (2016) 7:189–200. doi:10.1080/19490976.2015.1134082
125. Lyssiotis CA, Cantley LC. Acetate fuels the cancer engine. *Cell* (2014) 159:1492–4. doi:10.1016/j.cell.2014.12.009
126. Li L, Sun Y, Liu J, Wu X, Chen L, Ma L, et al. Histone deacetylase inhibitor sodium butyrate suppresses DNA double strand break repair induced by etoposide more effectively in MCF-7 cells than in HEK293 cells. *BMC Biochem* (2015) 16:2. doi:10.1186/s12858-014-0030-5
127. Gospodinov A, Popova S, Vassileva I, Anachkova B. The inhibitor of histone deacetylases sodium butyrate enhances the cytotoxicity of mitomycin C. *Mol Cancer Ther* (2012) 11:2116–26. doi:10.1158/1535-7163.MCT-12-0193
128. Koprinarova M, Botev P, Russev G. Histone deacetylase inhibitor sodium butyrate enhances cellular radiosensitivity by inhibiting both DNA nonhomologous end joining and homologous recombination. *DNA Repair (Amst)* (2011) 10:970–7. doi:10.1016/j.dnarep.2011.07.003
129. Shan YS, Fang JH, Lai MD, Yen MC, Lin PW, Hsu HP, et al. Establishment of an orthotopic transplantable gastric cancer animal model for studying the immunological effects of new cancer therapeutic modules. *Mol Carcinog* (2011) 50:739–50. doi:10.1002/mc.20668
130. Usami M, Kishimoto K, Ohata A, Miyoshi M, Aoyama M, Fueda Y, et al. Butyrate and trichostatin A attenuate nuclear factor kappaB activation and tumor necrosis factor alpha secretion and increase prostaglandin E2 secretion in human peripheral blood mononuclear cells. *Nutr Res* (2008) 28:321–8. doi:10.1016/j.nutres.2008.02.012
131. Kendrick SF, O'Boyle G, Mann J, Zeybel M, Palmer J, Jones DE, et al. Acetate, the key modulator of inflammatory responses in acute alcoholic hepatitis. *Hepatology* (2010) 51:1988–97. doi:10.1002/hep.23572
132. Chang PV, Hao L, Offermanns S, Medzhitov R. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proc Natl Acad Sci U S A* (2014) 111:2247–52. doi:10.1073/pnas.1322269111
133. Singh N, Thangaraju M, Prasad PD, Martin PM, Lambert NA, Boettger T, et al. Blockade of dendritic cell development by bacterial fermentation products butyrate and propionate through a transporter (Slc5a8)-dependent inhibition of histone deacetylases. *J Biol Chem* (2010) 285:27601–8. doi:10.1074/jbc.M110.102947
134. Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, et al. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. *Nat Med* (2014) 20:159–66. doi:10.1038/nm.3444
135. Wang X, Lin Y. Tumor necrosis factor and cancer, buddies or foes? *Acta Pharmacol Sin* (2008) 29:1275–88. doi:10.1111/j.1745-7254.2008.00889.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership