MUTANT P53 IN CANCER PROGRESSION AND PERSONALIZED THERAPEUTIC TREATMENTS

EDITED BY: Marco Cordani, Dawid Walerych, Jerson L. Silva, Oleg Timofeev and Mara Cirone

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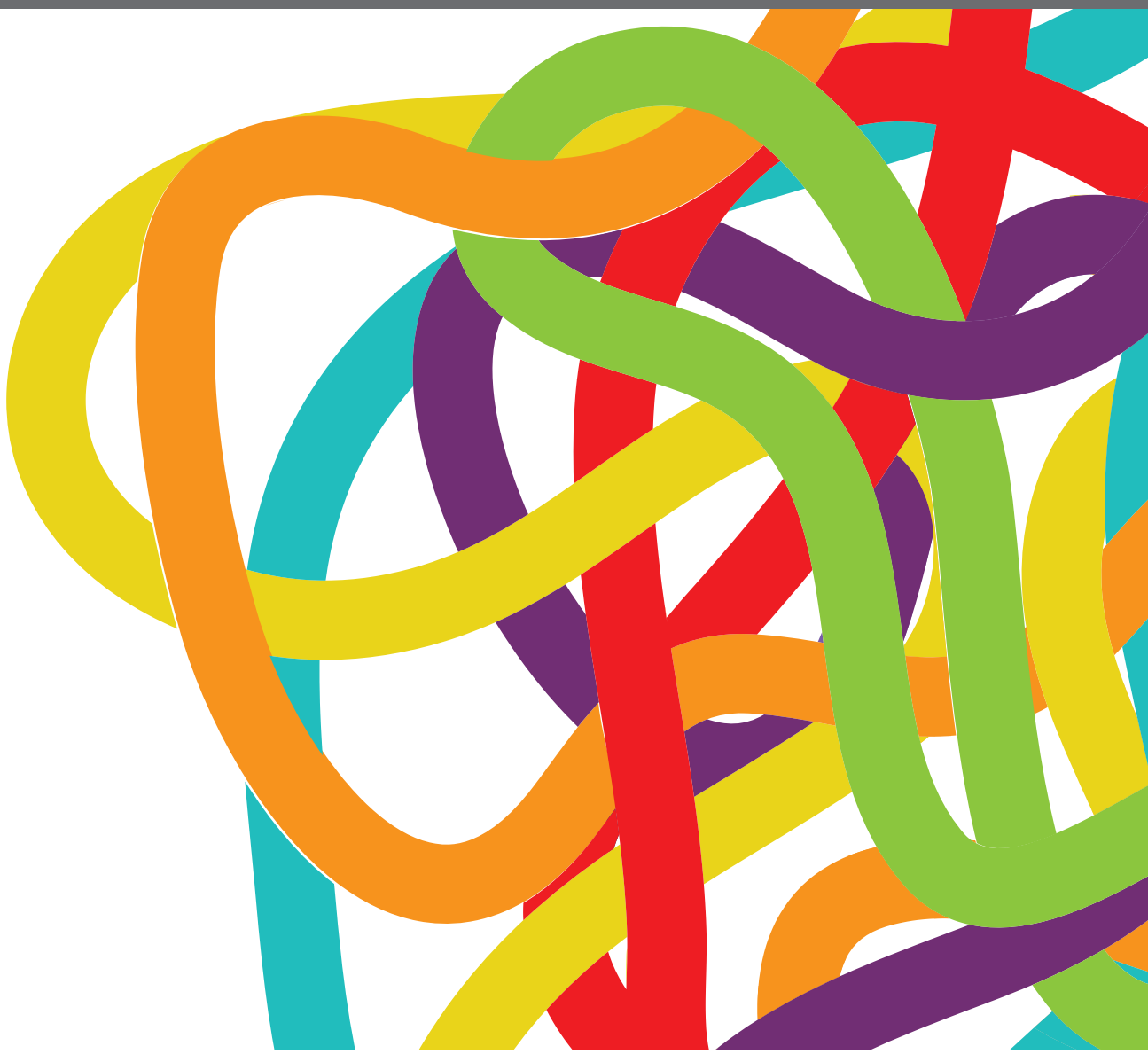

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MUTANT P53 IN CANCER PROGRESSION AND PERSONALIZED THERAPEUTIC TREATMENTS

Topic Editors:
Marco Cordani, Université Claude Bernard Lyon 1, France
Dawid Walerych, Polish Academy of Sciences, Poland
Jerson L. Silva, Federal University of Rio de Janeiro, Brazil
Oleg Timofeev, Philipps University of Marburg, Germany
Mara Cirone, Sapienza University of Rome, Italy

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EDITORIAL
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Editorial: Mutant p53 in Cancer Progression and Personalized Therapeutic Treatments

*Oleg Timofeev**

Institute of Molecular Oncology, Universities of Giessen and Marburg Lung Center (UGMLC), Member of the German Center for Lung Research (DZL), Philipps-University, Marburg, Germany

Keywords: mutant p53, targeted therapy, tumor stroma, metabolic reprogramming, cancer stem cell

Editorial on the Research Topic

Mutant p53 in Cancer Progression and Personalized Therapeutic Treatments

The p53 tumor suppressor is a transcriptional factor that controls a network of cellular processes essential for the maintenance of genomic integrity and prevention of malignant transformation. Ironically, p53 was initially described as an oncogene, and it took quite some time to realize that the protein found in tumors was a mutated version. Mutations in the TP53 gene are the most frequent genetic alterations detected in human cancers. In contrast to other tumor suppressors that are usually inactivated by frame-shift or nonsense mutations, the majority of cancer-associated TP53 mutations are non-synonymous missense substitutions, which indicate that cancer cells can benefit from the presence of mutated p53 protein with compromised functions (loss of function – LOF). Furthermore, some common TP53 missense mutations possess gain-of-function (GOF) properties that furnish mutant protein with new oncogenic qualities. A plethora of mutations detected in the TP53 gene, which gives rise to more than 2000 different protein variants, makes the issue very complex, but the progress in experimental and data mining methods provided significant advances in understanding functional consequences of different p53 mutations.

In this Research Topic, a few excellent reviews summarize the latest achievements in this field. Zhu et al. outline the spectrum of tumorigenic activities displayed by mutant p53 and discuss different therapeutic strategies to target cancer with TP53 mutations. The key targeted approaches – small molecule reactivators, gene editing, and immunotherapy aimed at mutant p53 tumors are also the topic of the review by Chasov et al. The work of Alvarado-Ortiz et al. is focused on the molecular mechanisms underlying the oncogenic activity of GOF p53 mutants. Based on the recent publications they analyze the role of p53 GOF mutations in metastasis, immune escape, metabolic reprogramming, cancer cell plasticity, and therapy resistance, and consider therapeutic perspectives. Metabolic functions of mutant p53 are particularly addressed by Etichetti et al. The authors recap current evidences for the role of mutant p53 in alteration of the mevalonate pathway and its contribution to enhanced prenylation of oncogenic proteins *via* positive regulation of Isoprenylcysteine Carboxyl Methyltransferase (ICMT). The interplay between mutant p53 and mevalonate pathway is further addressed in the research of Romeo et al. They explore how inhibition of STAT3 affects the mevalonate pathway and influences the expression of HSP90. The authors provide data arguing for the role of STAT3 in the stabilization of mutant p53 protein. On the other hand, it is known that in colorectal cancer the GOF hot-spot $p53^{R248Q/W}$ mutants can

Edited and reviewed by:
Tao Liu,
University of New South Wales, Australia

***Correspondence:**
Oleg Timofeev
o_timofeev@hotmail.com

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positively regulate STAT3 activity *via* direct binding and protecting it from inhibitory dephosphorylation. Klemke et al. dissect the role of these and other mutant p53 variants in pancreatic cancer. Using a panel of PDAC cell lines they uncover a specific function of the $p53^{R248W}$ mutant for enhancing the STAT3 axis which promotes migration of cancer cells. This study highlights p53 mutation-specific oncogenic features and underlines the functional heterogeneity of mutant variants. Hu et al. explore the role of TP53 mutations in PDAC using publically available gene expression data from patient samples. They uncover a link between mutant p53, small nuclear RNA (snoRNA)-mediated maturation of ribosomal RNA (rRNA), and PDAC progression: tumors with mutant p53 display increased snoRNA and rRNA levels, which correlates with poor prognosis. The relevance of TP53 mutational variability in cancer is in the focus of the review of Monti et al. The impact of p53 mutant variants for Chronic Lymphocytic Leukemia (CLL) is discussed here from the clinical perspective. Although TP53 mutations in CLL are rare and detected in less than 10% of cases at initial diagnosis, leukemia with compromised p53 displays faster progression and poor response to chemo- and immunotherapy. Unlike CLL, high-grade ovarian serous cancer (HGSOC) is characteristic of extremely high frequency (about 95%) and large variability of p53 mutations. Boyarskikh et al. analyze the spectrum of TP53 alterations in HGSOC patients with BRCA1/2 mutations. When looking for the association between BRCA1/2 and TP53 status the researchers find an unexpectedly increased proportion of TP53 truncations. Interestingly, almost 50% of detected p53 variants with missense mutations fall into a poorly characterized "unclassified" group with no clear LOF or GOF characteristics. p53 mutations affect not only classical tumor-suppressive mechanisms such as apoptosis or cell cycle arrest but can have an impact on other processes also relevant for tumor suppression. The role of wild-type p53 in the regulation of autophagy is well established, but it is less known how p53 mutations affect this process. Shi et al. provide a review of recent publications in this field and discuss how autophagy can be used for targeting mutant p53 in cancer. The latest progress in the understanding of the relationship between p53 activity and stemness is addressed in the review of Ghatak et al. The authors discuss how p53 controls pluripotency in normal cells and how oncogenic TP53 mutations drive cancer metastasis, cell plasticity, and resistance to therapy. Identification of mechanisms underlying chemoresistance associated with functional inactivation of p53 is an important task for the development of effective therapy. Deng et al. describe a p53-dependent mechanism that confers drug resistance in non-small lung cancer (NSCLC). Peroxisome proliferator-activated receptor coactivator (PGC1α) is essential for mitochondrial biogenesis and function, but its overexpression in NSCLC compromises the efficacy of platinum-based therapy and correlates with worse survival. Authors suggest that wild-type p53 is involved in the regulation of PGC1α protein stability, thus making cancer cells sensitive to chemotherapy. Tumor stroma can also contribute to drug resistance. Cancer cells induce changes in surrounding fibroblasts, endothelial and immune cells to adjust the microenvironment and make it favorable for tumor growth and dissemination. This is mediated by altered secretome and surface molecules produced by cancer cells. Besides cell-autonomous effects, p53 mutations can enhance the influence of cancer cells on the microenvironment. Capaci et al. provide a comprehensive review of recent publications dealing with the role of mutant p53 in shaping the tumor stroma.

Despite over 40 years of extensive research and myriad publications, our understanding of the role of p53 mutations in cancer development and therapy response is by far not complete. The papers collected in this Research Topic elucidate many important cellular mechanisms affected by p53 mutations and provide an outlook for the clinical consequences in different cancers. Together the reviews and experimental articles presented here have a significant impact on the field of p53 research.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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

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ORIGINAL RESEARCH
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STAT3 and mutp53 Engage a Positive Feedback Loop Involving HSP90 and the Mevalonate Pathway

Maria Anele Romeo[1,2†], *Maria Saveria Gilardini Montani*[1,2†], *Rossella Benedetti*[1,2], *Roberta Santarelli*[1,2], *Gabriella D'Orazi*[3,4] *and Mara Cirone*[1,2*]

[1] Department of Experimental Medicine, "Sapienza" University of Rome, Rome, Italy, [2] Laboratory Affiliated to Istituto Pasteur Italia-Fondazione Cenci Bolognetti, "Sapienza" University of Rome, Rome, Italy, [3] Department of Research, IRCCS Regina Elena National Cancer Institute, Rome, Italy, [4] Department of Medical, Oral and Biotechnological Sciences, University "G. d'Annunzio", Chieti, Italy

Oncosuppressor TP53 and oncogene STAT3 have been shown to engage an interplay in which they negatively influence each other. Conversely, mutant (mut) p53 may sustain STAT3 phosphorylation by displacing SH2 phosphatase while whether STAT3 could influence mutp53 has not been clarified yet. In this study we found that pharmacologic or genetic inhibition of STAT3 in both glioblastoma and pancreatic cancer cells, carrying mutp53 protein, reduced mutp53 expression level by down-regulating chaperone HSP90 as well as molecules belonging to the mevalonate pathway. On the other hand, HSP90 and the mevalonate pathway were involved in sustaining STAT3 phosphorylation mediated by mutp53. In conclusion, this study unveils for the first time that mutp53 can establish with STAT3, similarly to what observed with other oncogenic pathways, a criminal alliance with a crucial role in promoting cancerogenesis.

Keywords: STAT3, mutp53, HSP90, mevalonate kinase, glioblastoma, pancreatic cancer

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Simona Pisanti,
University of Salerno, Italy

Reviewed by:
Alejandro Parrales,
University of Kansas Medical Center Research Institute, United States
Massimo Donadelli,
University of Verona, Italy

***Correspondence:**
Mara Cirone
mara.cirone@uniroma1.it

†These authors have contributed equally to this work

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INTRODUCTION

Signal Transducer and Activator of transcription (STAT) 3 is emerging as one of the most promising therapeutic targets in anti-cancer therapy, as its phosphorylation, especially in 705 tyrosine (Tyr705) residue, strongly contributes to cancer survival, progression and chemo-resistance (1). As such, STAT3 inhibition has been reported to trigger apoptosis in a variety of hematological and solid cancers displaying constitutive STAT3 activation (2), also inducing an immunogenic cell death type (3, 4). STAT3 may be activated by cytokines such as IL-6 and IL-10 that, released by tumor cells, besides acting in an autocrine fashion to sustain cancer cell survival, may activate this pathway in myeloid immune cells in the tumor environment, resulting in an impairment of their function (5). Therefore, targeting STAT3 may have a double beneficial effect, as it can act on both cancer and immune cell side. STAT3 has been reported to inhibit the expression of wtp53 at transcriptional level, as site-specific mutation in the STAT3 DNA-binding site in the p53 promoter partially abrogates such effect (6). The finding that STAT3 activation inhibits wtp53 is not surprising, as STAT3 is a pro-oncogenic molecule while wtp53 acts as a tumor suppressor molecule whose function is incompatible with cancer development. For this reason, p53 results mutated in over 50% of cancers and inhibited in the majority of the remaining ones. In line with these findings, we have shown that the inhibition of the constitutive STAT3 phosphorylation by Apigenin activated wtp53 to reduce Primary Effusion Lymphoma cell survival (7). More recently AG490 STAT3 inhibitor, by activating the p53-p21 axis, has been found to trigger KSHV replication

in lymphoma cells harboring a latent viral infection (8). p53-p21 axis has been previously reported by ours and others' laboratories to be involved in KSHV lytic cycle activation (9, 10). Interestingly, other authors have reported that the wtp53 inhibited STAT3 activation, impairing its DNA binding activity (11), which suggests a reciprocal negative regulation between these two molecules. Less elucidated is the interplay between STAT3 and mutp53. This issue has been recently addressed by a study showing that, differently from wtp53, mutp53 sustained STAT3 phosphorylation by displacing the phosphatase SHP2 (12). However, whether STAT3 activation could influence mutp53 has not been explored yet. It is emerging that p53, as results of mutations that can occur at different sites, although more frequently in the DNA binding domain, not only loose its onco-suppressive capacity (LOS) but may also acquire oncogenic properties (GOF) (13). This is not due to the intrinsic characteristic of mutp53 but rather depends on its interaction with oncogenic pathways that mutp53 activates to increase its own stability. Among those, HSF1/HSP90 and the mevalonate pathways, both strongly involved in cancerogenesis (13). The HSF1/HSP90 pathway regulates the transcription of chaperoning molecules required for stabilization of oncoproteins and for helping cancer cells to cope with basal or induced cell stress (14) while the mevalonate pathway is needed for sterol and not-sterol isoprenoid production, required for post-translational modification of several proteins involved in cancerogenesis (15).

Interestingly, HSP90 (16) and molecules involved the mevalonate pathways (17) have been reported to be regulated by STAT3. Therefore, in the present study, we investigated whether STAT3 inhibition could reduce HSP90 and the mevalonate pathway molecules and through this mechanism reduce mutp53 expression level. As HSP90 and mevalonate pathway engage a cross-talk with mutp53 (13, 18), we then evaluated whether the downregulation of these molecules could contribute to the inhibition of STAT3 Tyr705 phosphorylation induced by mutp53 depletion. Finally, we evaluated whether STAT3 inhibition could impair cell survival and affect p53 and the mevalonate pathway in wtp53 carrying cancer cells.

MATERIALS AND METHODS

Cell Cultures and Treatments

U373, T98G, and U87 (human glioblastoma cell lines with mutant and wild type p53) and Panc1 (human pancreatic cancer cell line with mutant p53) were grown in RPMI 1640 (Thermo Fisher Scientific), 10% Fetal Bovine Serum (FBS) (Corning), L-glutamine, streptomycin (100 μg/ml) (Corning), and penicillin (100 U/ml) (Corning) in 5% CO_2 at 37°C. Cells were always detached using Trypsin-EDTA solution (Biological Industries, Cromwell, CT, USA).

U373, T98G, U87, and Panc1 cells were treated with AG490 (100 μM) (Millipore) for 48 h. U373 cells were treated with lovastatin (50 μM) (Sigma Aldrich) for 24 h. U373 cells were pre-treated with bortezomib (5 nM) (Santa Cruz Biotechnology) for 30 min and then treated with AG490 (100 μM) (Millipore) for 48 h. U373 and Panc1 cells were treated with geldanamycin (100 nM) (Sigma Aldrich) for 24 h.

Trypan Blue Exclusion Assay

U373, Panc1, T98G, and U87 cells were plated in 6-well plates at a density of 2×10^5 cells/well. The following day, when the cells were in the exponential growth phase, cells were treated with 100 μM AG490. After 48 h of culture, a trypan blue (Sigma Aldrich) exclusion assay was performed to test cell viability. Cells were counted by light microscopy using a Neubauer emocytometer. The experiments were performed in triplicate and repeated at least three times.

STAT3 and p53 Silencing

1.5×10^6 U373 cells were transfected with specific STAT3 siRNA transfection (Santa Cruz Biotechnology, sc-29493) and control siRNA-A (Santa Cruz Biotechnology, sc-37007) as a scrambled control for STAT3 knockdown, or with sip53 plasmid and empty vector as control for p53 knockdown, by electroporation using the Bio-Rad Pulse Controller at 180 V, according to the manufacturer's instructions, and cultured in 24 well plates. After 48 h cells were lysate and protein extracts were subjected to western blot analysis.

Western Blot Analysis

1×10^6 cells were washed with PBS and lysed in a RIPA buffer containing 150 mM NaCl, 1% NP-40 (Calbiochem), 50 mM Tris-HCl, pH 8, 0.5% deoxycholic acid (SIGMA), 0.1% SDS, protease and phosphatase inhibitors. Twelve microgram of protein lysates were subjected to protein electrophoresis on 4–12% NuPage Bis-Tris gels (Sigma Aldrich). The gels were blotted on nitrocellulose membrane (Biorad) for 1 h in Tris-Glycine buffer. The membranes were blocked in PBS 0.1% Tween20 solution containing 3% of BSA, probed with specific antibodies and developed using ECL Blotting Substrate (Advansta).

Antibodies

To evaluate the expression of proteins we used the following antibodies: mouse monoclonal anti-STAT3 (1:500) (BD Transduction Laboratories, 610,189), rabbit polyclonal anti-phospho-STAT3 (1:500) (p-Tyr705, clone D3A7, Cell Signaling Technology, 9145), mouse monoclonal anti-p53 (1:100) (clone DO-1, Santa Cruz Biotechnology Inc., sc-126), mouse monoclonal anti-HSP90 (1:100) (Santa Cruz Biotechnology Inc., sc-69703), mouse monoclonal anti-p21 (1:100) (clone F-8, Santa Cruz Biotechnology Inc., sc-271610), mouse monoclonal anti-SREBP1 (1:100) (clone A-4, Santa Cruz Biotechnology Inc., sc-365513), mouse monoclonal anti-MVK (1:100) (clone D-3, Santa Cruz Biotechnology Inc., sc-390669). Mouse monoclonal anti-β-actin (1:10,000) (Novus Biological, NB600-501) and goat polyclonal anti-lamin B (1:100) (Santa Cruz Biotechnology Inc, sc-374015) were used as loading control. The goat anti-mouse IgG-Horseradish Peroxidase (Santa Cruz Biotechnology Inc., sc- 2005), goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology Inc., sc-2004) and rabbit anti-goat IgG-HRP (Santa Cruz Biotechnology Inc., sc-2768) were used as

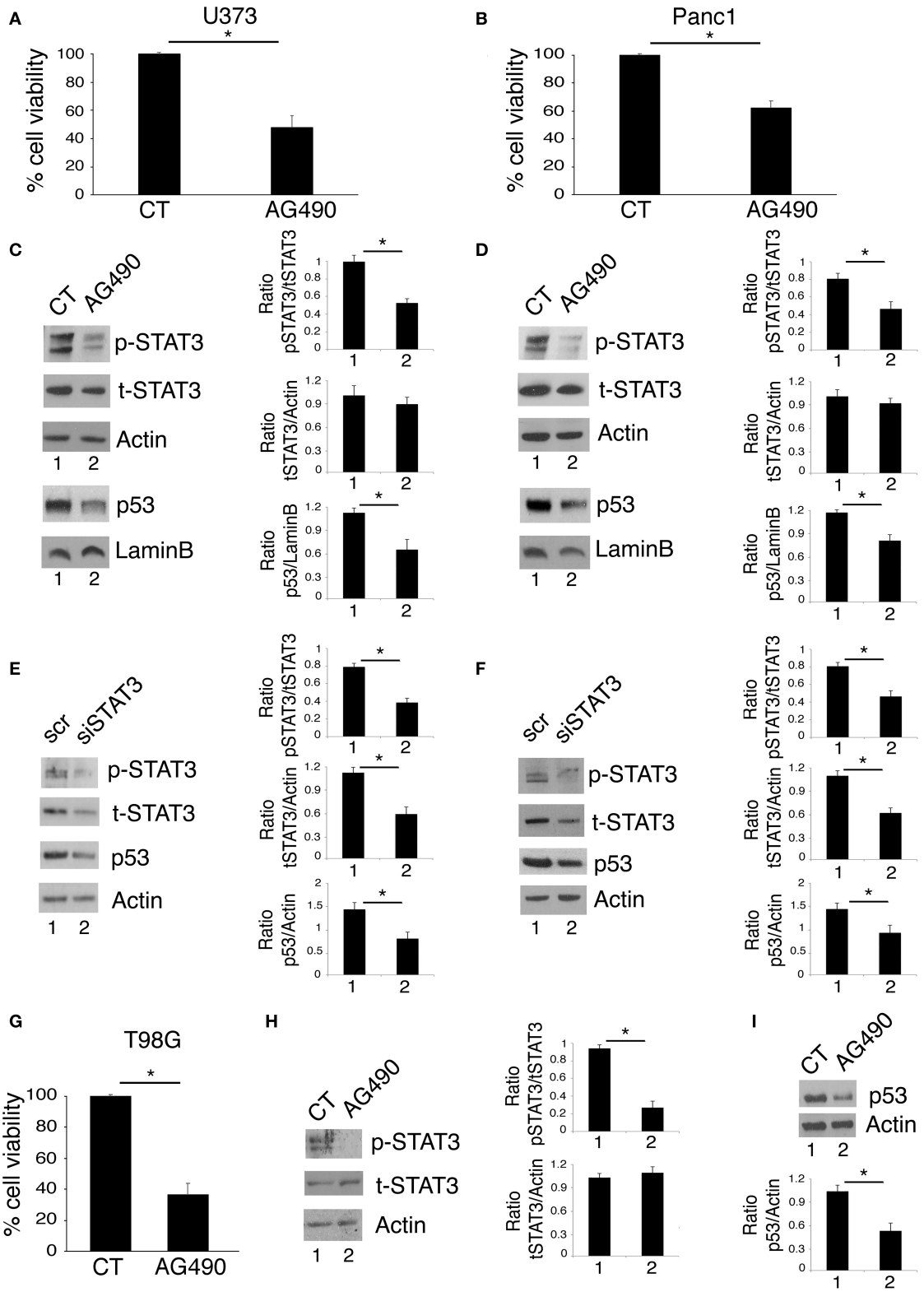

FIGURE 1 | STAT3 inhibition reduces cell survival and mutp53 expression in U373 and Panc1 cell lines. U373, Panc1, and T98G cell lines were cultured in the absence or in the presence of 100 μM AG490 for 48 h, and cell survival and mutp53 expression together with p-STAT3 and t-STAT3 expression were studied by

(Continued)

FIGURE 1 | trypan blue exclusion assay **(A,B,G)** and by western blot **(C,D,H,I)**, respectively. The histograms **(A,B,G)** represent the mean plus S.D. of more than 3 experiments $^*P < 0.05$; in western blot **(C,D)** Lamin B and **(H,I)** β Actin were used as loading control. In **(E,F)** mutp53 expression was evaluated by western blot in STAT3-silenced U373 **(E)** and Panc1 **(F)** for 48 h. β Actin was used as loading control. One representative experiment out of 3 is shown. The histograms represent the mean plus S.D. of the densitometric analysis of the ratio of specific band and control of 3 different experiments. $^*P < 0.05$.

secondary antibodies. All the primary and secondary antibodies were diluted in PBS-0.1% Tween20 solution containing 3% of BSA (SERVA).

Densitometric Analysis

The quantification of proteins bands was performed by densitometric analysis using the Image J software (1.47 version, NIH, Bethesda, MD, USA), which was downloaded from NIH website (http://imagej.nih.gov).

Measurement of Intracellular Reactive Oxygen Species Production

To measure reactive oxygen species (ROS) production, 2,7-dichlorofluorescein diacetate (DCFDA; Sigma-Aldrich D6883) 10 μM was added to cell cultures for 15 min and live cells gated according to their forward scatter (FSC) and side scatter (SSC) properties were analyzed by FACScalibur flow cytometer (BD Transduction Laboratories), using CELLQuest Pro software (version 6.0, BD Biosciences). For each analysis 10,000 events were recorded.

Statistical Analysis

Results are represented by the mean ± standard deviation (SD) of at least three independent experiments and a two-tailed Student's *t*-test was used to demonstrate statistical significance. Difference was considered as statistically significant when *p*-value was at least < 0.05.

RESULTS

Tyr705 STAT3 Inhibition by AG490 or STAT3 Silencing Reduce Cell Survival and mutp53 Expression Level in Glioblastoma and Pancreatic Cancer Cells Carrying R273 mutp53

Targeting STAT3 has been shown to be an effective strategy to reduce the survival of several aggressive cancers that display constitutive Tyr705 STAT3 activation and p53 mutations, including glioblastoma (19) and pancreatic cancers (20, 21). Accordingly, in this study we found that the treatment with AG490 JAK2/STAT3 inhibitor reduced cell survival in glioblastoma (U373) and pancreatic (Panc1) cancer cell lines, harboring R273 hot spot mutation in DNA binding domain of p53 (**Figures 1A,B**). As mutp53 carrying cells strongly relay on its expression for their survival, we investigated whether AG490-mediated cytotoxicity could correlate with the reduction of mutp53 expression level. We found that the inhibition of STAT3 phosphorylation by AG490 down-regulated mutp53 in both cell lines (**Figures 1C,D**) and that also STAT3 silencing by specific siRNA reduced mutp53 expression level in U373 and Panc1 cells (**Figures 1E,F**). Finally, we used T98G glioblastoma cells, carrying M237I mutp53 and found that AG490 reduced cell survival and mutp53 expression also in these cells (**Figures 1G–I**), suggesting a more general effect link between STAT3 phosphorylation and p53 mutations.

STAT3 Cross-Talks With the Mevalonate Pathway to Sustain mutp53 Expression Level

By gene expression profiling approaches, it has been identified that STAT3 regulates the expression of the sterol regulatory element-binding proteins (SREBPs) and the transcription of the mevalonate cascade enzymes (22). Interestingly, the mevalonate pathway plays an important role in mutp53 stability, in a positive feedback loop (13, 23). Based on this knowledge, we next investigated whether STAT3 inhibition could affect the mevalonate pathway to down-regulate mutp53. At this aim, the expression level of SREBP1, one of the most important transcription factors controlling the transcription of the mevalonate enzymes and MVK, a key kinase of the mevalonate pathway, were investigated in U373 and Panc1 cells treated with AG490. The results shown in **Figures 2A,B** show that AG490 down-regulated SREBP1 in both cell lines and that also MVK expression level was reduced by such treatment (**Figures 2C,D**). The importance of the mevalonate pathway in down-regulating mutp53 expression in AG490-treated U373 cells was supported by the use of lovastatin, an inhibitor of the mevalonate pathway that efficiently reduced mutp53 expression level in these cells (**Figure 2E**). Interestingly, we found that lovastatin also inhibited STAT3 phosphorylation (**Figure 2E**) suggesting the occurrence of a cross-talk between STAT3 and the mevalonate cascade.

STAT3/HSP90 Interplay Sustains mutp53 Expression Level and the Mevalonate Pathway

Previous studies by our and other's laboratories have shown that inhibition of STAT3 down-regulated HSP90 expression in cancer cells (2, 16). As mutp53 is highly dependent on HSP90 for its stability (23–25), we then investigated whether the reduction of mutp53 expression level induced by STAT3 inhibition could correlate with the down-regulation of HSP90. As shown in **Figures 3A,B**, HSP90 expression level was reduced by AG490 as well as by STAT3 silencing by specific siRNA. These results suggest that the reduction of mutp53 expression level mediated by STAT3 inhibition was involved the down-regulation of HSP90. To evaluate whether the reduction of mutp53 was due to its proteasomal degradation, we used the proteasome inhibitor

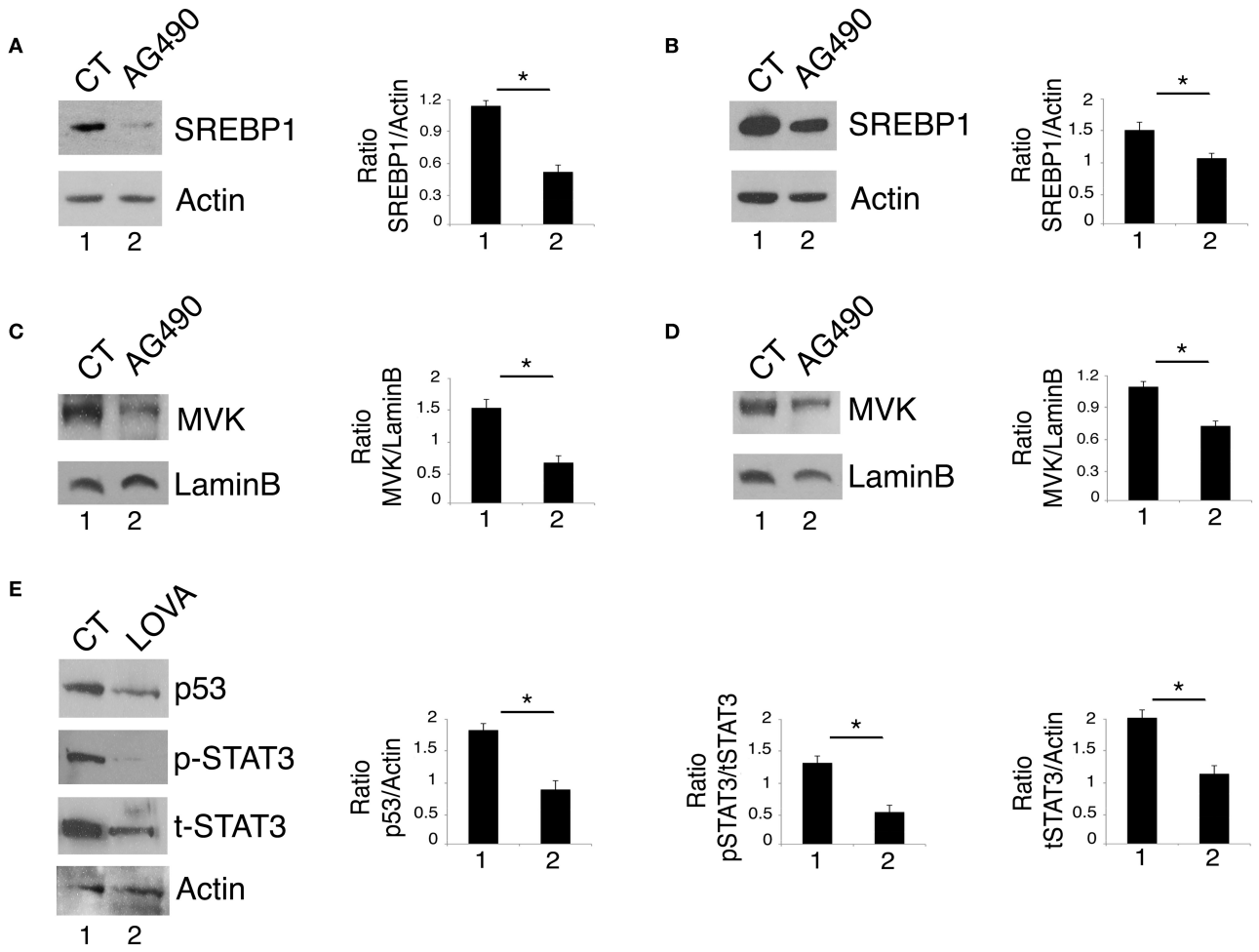

FIGURE 2 | STAT3 cross-talks with the mevalonate pathway and sustains its interplay with mutp53. U373 **(A,C)** and Panc1 cells **(B,D)** cultured with 100 μM AG490 were analyzed by western blot for SREBP1 and MVK expression. In **(E)**, mutp53 and STAT3 expression of U373 cells cultured for 24 h with 50 μM lovastatin (LOVA) was analyzed by western blot. β Actin and Lamin B were used as loading control. One representative experiment out of 3 is shown. The histograms represent the mean plus S.D. of the densitometric analysis of the ratio of specific band and control of 3 different experiments. *$P < 0.05$.

bortezomib and found that mutp53 accumulated when this drug was used in combination with AG490 (**Figure 3C**). Interestingly, bortezomib treatment, together with mutp53 also increased MVK expression level (**Figure 3C**), further highlighting the correlation between the two molecules previously observed (26). The role of HSP90 in the stabilization of mutp53 in this setting was then confirmed by use of geldanamycin an HSP90 inhibitor that strongly reduced mut53 expression level in both U373 and Panc1 cell lines (**Figures 3D,E**). Interestingly, geldanamycin reduced also STAT3 phosphorylation (**Figures 3D,E**), suggesting that HSP90 may in turn sustain STAT3 phosphorylation, in a positive feedback loop crucial for mutp53 stability. Furthermore, geldanamycin reduced MVK expression (**Figure 3F**) and, on the other hand, lovastatin down-regulated HSP90 (**Figure 3G**) highlighting another important loop sustained by STAT3 activation.

p53 Silencing Inhibits STAT3 Activation in Cancer Cells Carrying mutp53

As mutp53 has been reported to sustain Tyr705 STAT3 phosphorylation in colon cancer cells (12), here we evaluated whether the silencing of R273 mutp53 could reduce the constitutive Tyr705 STAT3 phosphorylation also in our experimental conditions. As shown in **Figure 4**, mutp53 knocking down reduced STAT3 activation in U373 cells, confirming results previously obtained in other cell types.

We then found that the silencing of mutp53 reduced HSP90 and MVK expression (**Figure 4**), according to the positive feedback loops reported to occur between mutp53 and HSP90 or mutp53 and the mevalonate pathway (23, 27). Therefore, HSP90 and MVK down-regulation induced by mutp53 could contribute to the inhibition of STAT3 phosphorylation, in addition to the previously reported

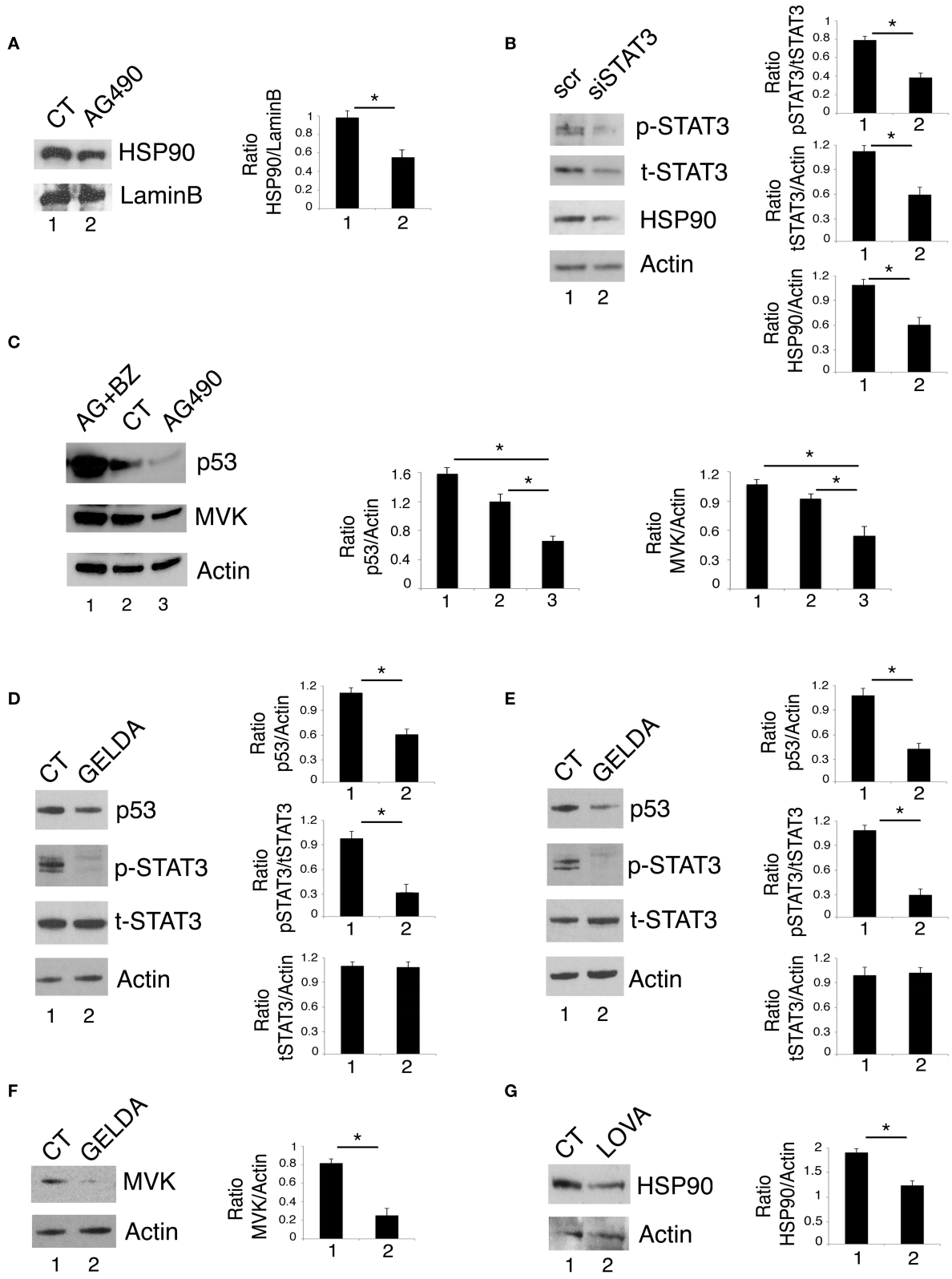

FIGURE 3 | STAT3/HSP90 interplay sustains mutp53 expression level. U373 cell line, cultured with 100 μM AG490 **(A)** or STAT3 silenced **(B)** for 48 h, was analyzed by western blot for HSP90 protein expression. Lamin B and β Actin were used as loading control. In **(C)**, U373 cells pre-treated or not with 5 nM of bortezomib (BZ)

(Continued)

FIGURE 3 | and then treated with 100 μM AG490 for 48 h were analyzed by western blot for the expression of p53 and MVK. In **(D–F)** U373 **(D,F)** and Panc1 **(E)** cells were cultured with 100 nM geldanamycin (GELDA) and the expression of mutp53, STAT3, and MVK **(F)** was evaluated by western blot. In **(G)**, the expression of HSP90 in U373 cells cultured for 24 h with 50 μM lovastatin (LOVA) was analyzed by western blot. β Actin was used as loading control. One representative experiment out of 3 is shown. The histograms represent the mean plus S.D. of the densitometric analysis of the ratio of specific band and control of 3 different experiments. *$P < 0.05$.

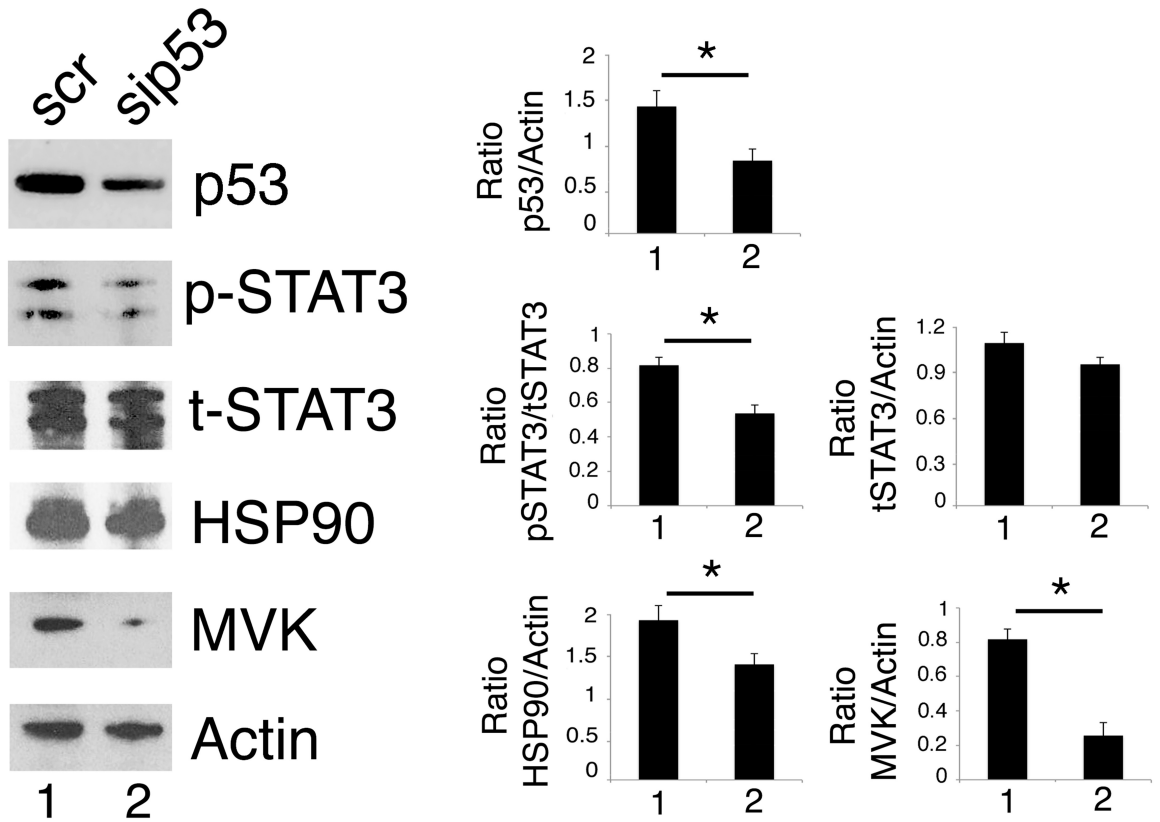

FIGURE 4 | p53 silencing inhibits STAT3 and reduces HSP90 and MVK expression level. U373 cells silenced for p53 for 48 h were analyzed by western blot to evaluate mutp53, STAT3, HSP90, and MVK protein expression. β Actin was used as loading control. One representative experiment out of 3 is shown. The histograms represent the mean plus S.D. of the densitometric analysis of the ratio of specific band and control of 3 different experiments. *$P < 0.05$.

displacing of SH2 phosphatase (12). The above reported results indicating that geldanamycin (**Figures 3D,E**) or lovastatin (**Figure 2E**) were able to reduce Tyr705STAT3 phosphorylation, supporting the possibility that the reduction of HSP90 and MVK could be involved in STAT3 inhibition mediated by mutp53 depletion.

STAT3 Inhibition Reduces Cell Survival by Activating p53 and Down-Regulating MVK in Glioblastoma Cancer Cells Carrying wtp53

We next investigated whether AG490 STAT3 inhibitor could exert cytotoxic effects against U87 glioblastoma cells harboring wtp53. We found that such treatment reduced cell survival also in these cells (**Figure 5A**) and inhibited STAT3 phosphorylation (**Figure 5B**). However, differently from mutp53, AG490 up-regulated wtp53 (**Figure 5C**) and its target p21 (**Figure 5D**) in this cell line. Moreover, we found that AG490 treatment slightly up-regulated HSP90 in U87 cells (**Figure 5E**), effect that could contribute to p53 stabilization. We then asked HSP90 up-regulation in U87 cells could correlate with an increase of ROS in U87 cells undergoing AG490 treatment. As shown in **Figure 5G**, we found that intracellular ROS increased in U87 wtp53 carrying cells while decreased in mutp53 carrying cells undergoing AG490 treatment. Next, as oppositely from mutp53, wtp53 has been reported to inhibit the mevalonate pathway, we then evaluated whether the activation of wtp53 by AG490 could correlate with a reduced expression of MVK in U87 cells. As shown in **Figure 5F**, we found that MVK expression was reduced in U87 cells treated with AG490 suggesting that the reduction of the mevalonate pathway could play a role in the impairment of cell survival induced by AG490 that increased wtp53 expression level.

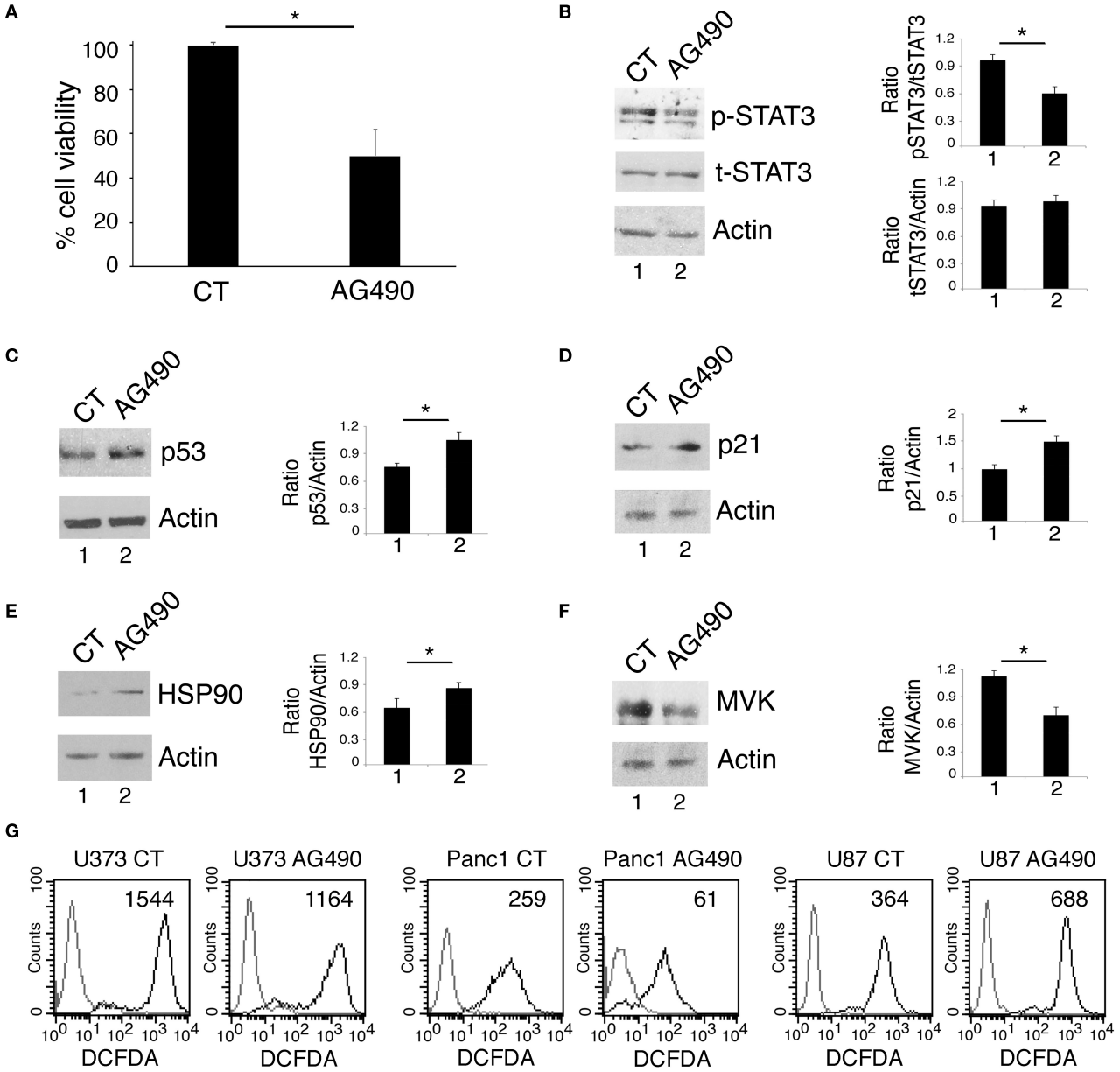

FIGURE 5 | STAT3 inhibition in wtp53 U87 cells reduces cell survival and increase p53 and p21 expression and inhibits the mevalonate pathway. U87 cells were cultured in the absence or in the presence of 100 μM AG490 for 48 h, and cell survival and STAT3, p53, p21, HSP90, and MVK expression were analyzed, respectively, by trypan blue exclusion assay **(A)** and by western blot **(B–F)**. The histograms **(A)** represent the mean plus S.D. of more than 3 experiments $^{*}P < 0.05$; in western blot **(B–F)** β Actin was used as loading control. One representative experiment out of 3 is shown. The histograms represent the mean plus S.D. of the densitometric analysis of the ratio of specific band and control of 3 different experiments. In **(G)**, FACS analysis of ROS production, by U373, Panc1, and U87 treated or not with 100 μM AG490, measured by DCFDA staining. The mean of fluorescence intensity is indicated. Solid gray peaks represent the controls. One representative experiment out of 3 is shown.

DISCUSSION

This study unveils for the first time that STAT3 sustains mutp53 expression level due to its interplay with HSP90 and the mevalonate pathway that increases its stability and prevents its degradation via proteasome. Previous studies have highlighted that HSP90 and the mevalonate cascade could increase mutp53 stability (13, 18, 23, 27, 28). Moreover, STAT3 has been reported to positively regulate HSP90 (2, 16) and SREBPs as well as several enzymes of the mevalonate pathway, including the mevalonate kinase MVK (22, 29, 30). Interestingly, HSP90 may in turn stabilize STAT3 (31) and maintain it phosphorylated by up-regulating the expression of JAKs, the most important kinases involved in Tyr705 STAT3 phosphorylation (32). The

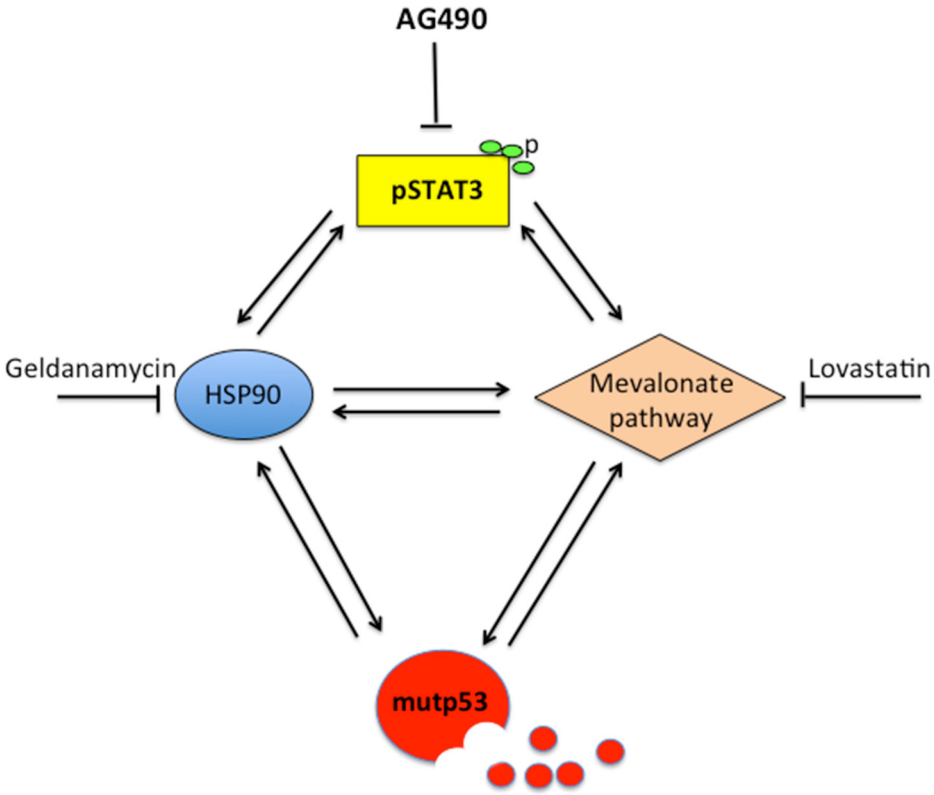

FIGURE 6 | Scheme showing the capacity of STAT3 to regulate HSP90, the mevalonate pathway, mutp53 and the cross-talk between these molecules.

mevalonate pathway may also contribute to STAT3 Tyr705 phosphorylation, as indicated by the use of statins that reduced STAT3 activation (17). In the present study, we found that the expression of HSP90 and MVK was down-regulated by p53-silencing, suggesting that STAT3 inhibition by mutp53 could involve the down-regulation of these molecules. This hypothesis was supported by the finding that geldanamycin or lovastatin reduced STAT3 phosphorylation in both U373 and Panc1 cells. This could be a new mechanism involved in the regulation of STAT3 by mutp53, in addition to the displacement of STAT3 phosphatase SHP2, recently reported (12). More importantly, this study suggests that STAT3 and mutp53 establish another criminal alliance that promotes cancerogenesis. The interplay with pro-oncogenic pathways is fundamental for mutp53 stability and GOF. Besides HSF1/HSP90 and mevalonate, indeed mutp53 engages a positive feed-back loop with NRF2, the most important transcription factor regulating the antioxidant response, and with HIF, essential for the adaption of cancer cells to hypoxia conditions (13, 23). The cross-talk with STAT3, highlighted in this study, suggests that STAT3 could another pathway crucial for mutp53 GOF. STAT3 is indeed able to up-regulate a variety of molecules involved in cell proliferation and evasion from apoptosis, including c-myc, survivin, and cyclin D (33, 34). STAT3 has been previously reported to positively influence the mevalonate (22, 29, 30) and HSF1/HSP pathways (16). Therefore, based on this and previous studies, STAT3 can be consider at the center of a hub crucial for the control of tumorigenesis (**Figure 6**). Indeed, STAT3 activation sustains the interplay between HSP90 and the mevalonate cascade, according to previous studies showing that HSF1 sustained the mevalonate pathway (35), that HSP90 sustained SREBP activation (36) and that Simvastatin inhibited HSP90 (37).

Of note, STAT3 can be activated by several cytokines including VEGF, whose production is promoted by STAT3 activation (38) and also by mutp53 due to its interaction with NFkB (13). Therefore, the interplay between STAT3 and mutp53 could play a pivotal role to induce a pro-cancerogenic microenvironment. Interestingly, several pro-inflammatory cytokines, acting in an autocrine fashion on cancer cells, besides STAT3, may activate other pro-oncogenic pathways such as mTOR, crucial for cancer survival (39). The relation between mTOR and mutp53 has also been previously investigated by studies showing that mutp53 could activate mTOR to inhibit autophagy and prevent its own degradation (40). STAT3 could cooperate with mTOR in preventing mutp53 degradation by autophagy. In conclusion, differently from other drugs that preferentially kill mutp53 carrying cancer cells (41), we found that the cytotoxic effect of STAT3 inhibition goes behind its-capacity to down-regulate mutp53, as AG490 efficiently impaired cell

survival also of wtp53 carrying glioblastoma cells, in correlation with p53 activation and the mevalonate pathway inhibition. Therefore, this study strongly encourages the targeting of STAT3 in anti-cancer therapy considering that, in addition to the inhibition of pro-survival molecules so far reported, our study suggests that STAT3 stabilizes mutp53 in cancer cells carrying mutp53 and activates wtp53 in cancer cells harboring wtp53.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

MR, MG, GD'O, and MC: design, acquisition, analysis, and interpretation of data. MC: writing manuscript. MG and MC: editing manuscript. MR, MG, RB, and RS: methodology. All authors: revised the drafts and approved the manuscript submission.

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Spectrum of *TP53* Mutations in BRCA1/2 Associated High-Grade Serous Ovarian Cancer

Ulyana A. Boyarskikh[1], L. F. Gulyaeva[2,3], A. M. Avdalyan[4], A. A. Kechin[1], E. A. Khrapov[1], D. G. Lazareva[5], N. E. Kushlinskii[6], A. Melkonyan[7], A. Arakelyan[7] and Maxim Leonidovich Filipenko[1]*

[1] Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences (SB RAS), Novosibirsk, Russia, [2] Institute for Medicine and Psychology, Novosibirsk State University, Novosibirsk, Russia, [3] Institute of Molecular Biology and Biophysics, Federal Research Center of Fundamental and Translational Medicine, Novosibirsk, Russia, [4] City Clinical Hospital No. 40, Moscow, Russia, [5] Altai Territorial Cancer Control Center, Barnaul, Russia, [6] N.N. Blokhin National Medical Research Center of Oncology, Moscow, Russia, [7] Group of Bioinformatics, Institute of Molecular Biology, Armenian National Academy of Sciences (NAS RA), Yerevan, Armenia

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Edited by:
Marco Cordani,
IMDEA Nanociencia, Spain

Reviewed by:
Alvaro Monteiro,
Moffitt Cancer Center, United States
Giovanni Gaudino,
Retired, Bellinzona, Switzerland

***Correspondence:**
Ulyana A. Boyarskikh
boyarskih.u@gmail.com

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Objective: Mutations in TP53 lead to loss of function (LOF) or gain of function (GOF) of the corresponding protein p53 and produce a different effect on the tumor. Our goal was to determine the spectrum of somatic *TP53* variants in *BRCA1/2* associated high-grade serous ovarian cancer (HGSOC).

Methods: The population under study comprised of HGSOCs with pathogenic variants in *BRCA1* ($n = 78$) or *BRCA2* ($n = 21$). Only chemo-naive and platinum-sensitive patients were included in this study. The case group of the IARC database ($n = 1249$) with HGSOC not stratified by BRCA status was used as a reference. A custom NGS panel was used for sequencing *TP53* and mutational hot-spots of other genes, and p53 expression was evaluated by immunohistochemistry for 68 cases of HGSOCs.

Results: Somatic *TP53* variants (95) or inhibition of wild-type p53 expression (3) were observed in 98 cases. The sample with normal p53 had *CDKNA1* variants. The frequency of truncating variants was significantly higher than in the reference cohort (30.3 vs. 21.0%, $p = 0.01$). Most of the samples (41/68) demonstrated low (or absent) expression of p53, and 17 samples overexpressed p53. LOH was typical for TP53 nonsense variants (14/15). In total, 68/95 samples were LOH positive and showed LOH in all tumorous cells, thus indicating the driver effect of *TP53* mutations. Three specimens had *KRAS*, *BAX*, *APC*, and *CTNNB1* subclones variants.

Conclusion: High frequency of *TP53* truncating variants, the low expression of mutant p53, and low incidence of oncogene mutations show potential GOF properties of p53 to be poorly represented in BRCA1/2 associated HGSOC.

Keywords: TP53 somatic mutations, p53 expression, gain of function, loss of function, *BRCA1/2 carriers*, ovarian cancer

INTRODUCTION

TP53 is the most frequently mutated gene in human cancer (1). About 75% of TP53 variants are missense substitutions (2). Other alterations include frameshift indels and nonsense variants (20%) and infrequent substitutions in splicing sites, inframe indels, and silence variants. Frameshift indels and nonsense variants always lead to the null p53 phenotype. In contrast, missense variants result in a full-size mutant p53, which can stably express and have a different effect on the tumor. The loss or decrease in p53 transcriptional activity (loss of function, LOF) is a common property of all p53 mutants associated with cancer. Along with LOF, a number of additional p53 action scenarios are possible. Specifically, variants of "separation of functions" are described (3). In this case, the mutant retains some pro-survival functions and selectively loses tumor-suppressive activity of p53 wild-type, as shown for the apoptosis-deficient R175P mutant p53 (4). Finally, some mutants acquire new oncogenic properties (gain of function, GOF). Studies of cancer cell lines and animal cancer models have shown that GOF TP53 variants can contribute to chemotherapy resistance and cancer progression (3, 5–8). Thus, mutant p53 plays a complex role in tumorigenesis that varies depending on both the mutation type and tumor origin.

The prevalence of missense variants and the fact that GOF mutants of TP53 promote tumor progression led to speculation about the positive selection of GOF variants during carcinogenesis.

High-grade serous ovarian carcinoma (HGSOC) is the most common type of ovarian cancer characterized by difficulties in early detection and high mortality rates. HGSOC is a unique type of cancer in terms of the prevalence of TP53 mutations. Almost all HGSOC tumors (95%) carry somatic TP53 variants. A comparable incidence of TP53 mutations is observed only in serous endometrial carcinomas (89%) and basal subtype breast tumors (88%) (1, 9). Almost 20% of HGSOCs are associated with germline BRCA1/2 variants (10). BRCA proteins is involved in the repair of DNA double-strand breaks by homologous recombination (HR) (11). The complete loss of BRCA1/2 function leads to disruption of the HR-based DNA repair and, as a result, to the large-scale genomic instability (12). In this case, alterations in TP53 (or other cell cycle control genes) are mandatory for the cell viability. Otherwise, genomic instability results in cell-cycle arrest and apoptosis. For HGSOC with an HR deficiency, we assumed LOF of p53 to be selected more frequently than GOF. To test this hypothesis, we determined the frequencies of various types of TP53 variants in the cohort of BRCA1/2-deficient HGSOCs. To evaluate the involvement of other genes in BRCA1/2-associated HGSOC pathogenesis, we tested these tumors for the frequent somatic variants in oncogenes.

MATERIALS AND METHODS

Patients and Samples

Samples were obtained from patients with relapse of high-grade serous ovarian adenocarcinomas after first-line treatment, undergoing tumor testing under the program "Improving the system of molecular genetic diagnosis of cancer in the Russian Federation" (http://www.cancergenome.ru). Ethical approval for this project was obtained from the Institute of Molecular Biology and Biophysics Ethics Committee (Protocol: # 1 dated March 14, 2017). Written informed consent was obtained from all subjects.

All patients underwent primary cytoreductive surgery, followed by adjuvant platinum-based chemotherapy as first-line treatment. Eligibility criteria for the study were (1) histologically verified diagnosis of high-grade serous ovarian (or fallopian tube) adenocarcinomas; (2) lack of neo-adjuvant therapy; (3) platinum sensitivity (the time from adjuvant platinum-based treatment to cancer relapse (platinum-free interval, PFI) was > 6 months), (4) available blood sample and FFPE tissue from the primary tumor; and (5) germline pathogenic variants in *BRCA1* or *BRCA2*. The FFPE primary tumor blocks were sectioned and stained with hematoxylin and eosin (H&E). Tumor regions on H&E stained slides were marked, and the percentage of tumor cells was estimated. Samples containing at least 10% of tumor cells were selected for this study. DNA was extracted from marked regions separated by manual macrodissection from three unstained 10 μm-thick FFPE sections. DNA isolation was carried out using alkaline lysis followed by DNA extraction from the precipitate, as described previously (13). DNA from blood leukocytes was extracted using an in-house method involving cell lysis using 10% SDS-containing buffer, proteinase K treatment, protein extraction using phenol-chloroform, and isopropanol precipitation of the DNA. All DNA samples were screened for sufficient quantity using the PCR based QC Kit (Kapa Biosystems). Germline *BRCA1/2* variants were determined by NGS of gene coding sequences and splicing site regions, as described previously (14). DNA samples from both leukocyte and FFPE tumors were sequenced. As a result, 99 tumors were revealed, with 78 being *BRCA1* germline mutation carriers and 21 – *BRCA2* (the full list of the BRCA variants is given in the **Supplementary Table 1**).

NGS Panel and Data Analysis

DNA target sequencing was performed using the PCR-based custom NGS panel called CCMSeq (Common Cancer Mutations). CCMSeq panel was designed for analyzing multiple genome regions that are commonly mutated in a variety of cancer types. This panel covers 8.6 kilobases across all 11 exons and adjacent intron regions of *TP53*, as well as coding regions of cancer-related genes that carry the most frequent somatic variants. These regions were selected based on the whole genome (or exome) sequencing data cataloged in the COSMIC database for colon, stomach, lung, breast, and ovary cancers. Regions carrying somatic mutations with a prevalence of at least 2% in two or more cancer types were included in the CCMSeq panel. In total, loci of 50 genes were selected for the CCMSeq panel (**Supplementary Table 2**). These selected targets were amplified using two multiplex PCRs with the amplicon library preparation procedure described previously (14). Normalized amplicon libraries were sequenced on a MiniSeq platform (Illumina) using a MiniSeq High Output Reagent Kit (300 cycles).

The procedure of NGS data analysis was similar to those described previously (14) with some modification: short-variant calling was performed by Pisces (https://github.com/Illumina/

Pisces, somatic mode). To filter the false positive variants, those with less than six reads of alternative alleles were discarded. The coverage (median coverage of one library amplicon) ranged from 163 to 7273 reads, with median and Q1-Q3 values of 1087 and 735-2037 reads, respectively. The following variant types were taken into consideration: (1) frameshift, stop gained, stop lost, start lost, splice acceptor, splice donor variants; (2) missense and splice region variants that according to 1000Genomes project have a population frequency less than 0.5%; (3) variants listed in the COSMIC database; (4) variants registered in the ClinVar database as "Pathogenic"/"Likely pathogenic." Differentiation between somatic and germline variants was performed by sequencing both leukocyte and tumor DNA samples. The variants with low variant allele frequency (VAF) were filtered out. The cut-off of VAF was 5% - for tumor DNA and 20% - for leukocyte DNA. DNA samples with somatic variant VAF <15% were sequenced twice.

The NGS data supporting this study have been deposited in the National Center for Biotechnology Information (NCBI)'s Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra) with BioProject ID PRJNA612603 and can be accessed at https://www.ncbi.nlm.nih.gov/sra/PRJNA612603.

Classification of TP53 Variants

TP53 variants were classified into three categories: GOF, LOF, and "unclassified," according to the criteria described by Brachova et al. (15). Specifically, *TP53* variants were defined as GOFs, based on experimental studies that showed the oncogenic properties of mutant p53. Eight *TP53* mutations were considered as GOF: P151S, Y163C, R175H, L194R, Y220C, R248Q/W, R273C/H/L, R282W. Nonsense and frameshift variants leading to significant disruptions in the p53 translation were classified as LOF. The remaining missense and splice site variants, the function of which is not yet well known, were categorized as "unclassified" variants.

Additionally, we used the International Agency for Research on Cancer (IARC) *TP53* database to further characterize all missense variants by the transcriptional and GOF activity in corresponding mutants (http://p53.iarc.fr/DownloadDataset.aspx (Files: somatic Mutation Data IARC *TP53* Database, R20.txt, and functional Assessment IARC *TP53* Database, R20.txt)). Characterization of GOF missense variants was performed, as described previously (16). As a reference group of variants specific for HGSOC the variant set of the IARC *TP53* database (File: somatic Variant Data IARC *TP53* Database, R20.txt) non-stratified by *BRCA* status, was used. Only the cases with morphology corresponding to adenocarcinoma and cystadenocarcinoma (1249 in total) were selected. Ethnicity and BRCA status were not indicated for most samples. The reference set of *TP53* variants did not contain silent variants.

Pathomorphological and Immunohistochemical Assessment

The percentage of tumor cells relative to other cells was estimated independently by two pathologists using the same slides stained with hematoxylin and eosin. In six tumors with extensive inflammatory infiltration and/or diffuse stromal invasion, epithelial cells were labeled with a pan-cytokeratin antibody cocktail (antibody clone AE1/AE3, M3515, Dako, CA, USA) for a more accurate estimation of tumor cell percentage. Finally, the percentage of tumor cells was calculated as the average value of both measurements. The mean difference between the measurements was $6 \pm 4\%$. The percentage of tumor cells ranged from 10 to 95% with the median and Q1-Q3 values of 60% and 45–75%, respectively (the actual percentage of tumor cells is given in **Supplementary Table 1**). FFPE tissue sections were subject to immunohistochemistry for p53 using a commercially available mouse monoclonal anti-human p53 antibody (clone DO-7, M7001, Dako, CA, USA) at the dilution of 1:50. Staining was performed on a whole section using a Ventana BenchMark GX autostainer (Roche) according to the standard protocol in automatic mode. Stained slides were examined by an experienced surgical pathologist who was not aware of molecular data. The percentage of cells with positive nuclear staining was estimated and subdivided into three categories: $\geq$ 70% positively stained nuclei (High); >10% and < 70% stained nuclei (Intermediate); $\leq$ 10% positively stained nuclei (Low).

Statistical Methods

Statistical analyses were performed applying R 2.13.1 statistical software. Results were compared using the χ^2 test.

RESULTS

Patient Characteristics

Ninety nine patients with HGSOC (4 with fallopian tube carcinoma and 95 with ovarian carcinoma) were enrolled in this study. In most patients (67/99, 67.0%), ovarian cancer was diagnosed at the age > 50 years. The median and Q1-Q3 values of age were 54 (49-60) years. The cohort of patients with HGSOC comprised cases with II (15), (III) 71, and IV (13) FIGO stages. Tumor grade was determined as G2 in 19 cases, G3 in 73 cases, and undetermined for others cases. All 99 patients underwent primary cytoreductive surgery, followed by first-line platinum-based chemotherapy, most with carboplatin-paclitaxel regimen (88). Other treatments included cisplatin-doxorubicin-cyclophosphamide (5), or not specified (platinum-based) (6). All patients experienced a complete or partial response after adjuvant therapy with platinum-free interval (PFI) > 6 months. For 32/99 patients, PFD was specified and ranged from 7 up to 21 months (median (Q1-Q3), 13 (11-16) months). All patients were carriers of a germline pathogenic BRCA1 or BRCA2 variants (78 and 21 cases, respectively). BRCA1 variants (5382insC, 300T/G, 4153delA, 2080delA, 3819del5, and 3875del4), highly prevalent in Slavs, accounted for 47.4% of total; full list of the BRCA variants is given in the **Supplementary Table 1**.

Repertoire and Distribution of Somatic Variants in HGSOC

FFPE primary tumors from 99 patients with relapse of BRCA-deficient ovarian cancer and paired blood samples were sequenced using CCMSeq panel. The detected somatic variants were absent in paired blood samples. Only somatic variants affecting the amino acid sequence of a protein were considered. A total of 106 somatic variants were identified across six genes

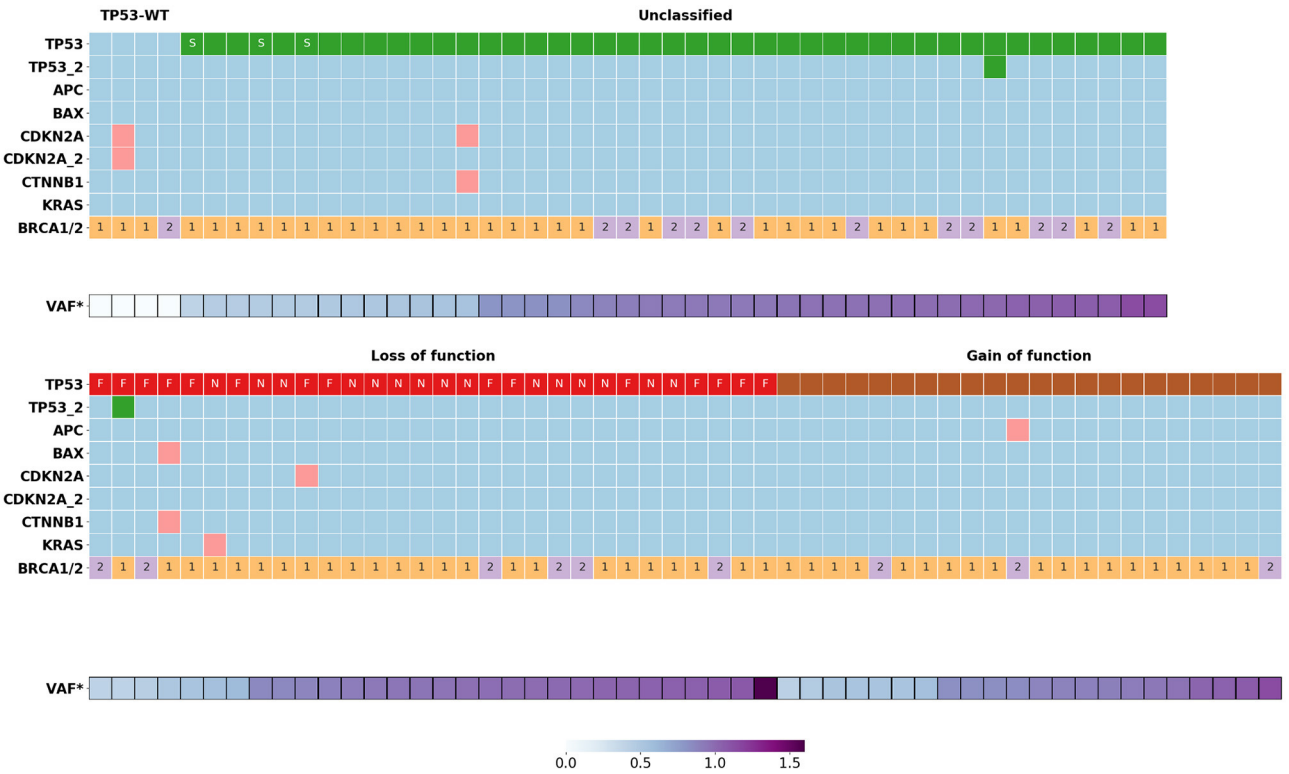

FIGURE 1 | Somatic mutations distribution in *BRCA1/2* associated HGSOC ($n = 99$). VAF*, normalized variant allele frequency of mutant *TP53* allele; S, variants of donor/acceptor splice sites; F, frame shift mutations; N, nonsense mutations; 1, *BRCA1* mutations; 2, *BRCA2* mutations.

(*TP53*, *APC*, *BAX*, *KRAS*, *CDKN2A*, *CTNNB1*) in 96/99 patients (see the variants destination and ClinVar classification in the **Supplementary Table 1**).

TP53 variants were found in 95 patients with HGSOC. In two cases, the tumor cells carried two *TP53* variants. *TP53* variants were grouped into three categories: GOF (22), LOF (30), and "unclassified" (45) (**Figure 1**, **Table 1**). We compared the results with the distribution of GOF, LOF and "unclassified" variants in the HGSOC variant set of the IARC TP53 database. The frequency of LOF variants (261/1249, 21%) in this group was significantly lower ($p < 0.01$).

There were no significant differences between the distribution of various types of *TP53* variants in BRCA1- and BRCA2-deficient HGSOC. Some of the "unclassified" *TP53* variants (16/45) were annotated in the ClinVar database as pathogenic, the functional significance of the remaining variants unknown. Most of the "unclassified" variants (38/45) were amino acid substitutions located in the DNA-binding domain (DBD, 34 variants), C-terminal tetramerization domain (2 variants) or interdomain spacer (residue 93 between Transactivation Domain and DBD, 2 variants). The rest of the "unclassified" variants were those of donor/acceptor splice sites (3), of splice regions (3) and disruptive inframe deletion (1).

The majority of variants (58/97) identified in *BRCA1/2* associated HGSOC were "unclassified" and GOF missense. We used the IARC database information to further characterize all missense variants by the presence of transcriptional and GOF activity in the corresponding mutants. In respect to transcriptional activity, the common missense variants (54/58, 93.1%) were classified as "nonfunctional," with the rest being "partially functional" (3) and "functional" (1). Mutants with preserved transcriptional activity were from the "unclassified" group. For most *TP53* variants, there were no data on their GOF properties. Petitjean et al. systematized information from the IARC database on GOF properties of 103 TP53 mutants (16). The authors identified three categories of GOF activity: (1) interference with p73, (2) transactivation of genes repressed by wild-type p53, and (3) cooperation with oncogenes for the transformation of mouse embryonic fibroblast cells. In our study, a variant was classified as GOF if the corresponding mutant had at least one of the GOF activities. According to these criteria, 6 of 43 "unclassified" missense variants were determined as GOF.

Six samples had nine concomitant variants in non-*TP53* genes such as *APC* (1), *BAX* (1), *KRAS* (1), *CDKN2A* (4), *CTNNB1* (2) (**Figure 1**, **Supplementary Table 1**). There was no significant association between the occurrence of these mutations and the clinical outcome of the corresponding patients, possibly due to the small size of this patient group.

Loss of Heterozygosity of TP53

For samples carrying somatic variants, we determined the percentage of tumor cells with loss of heterozygosity (LOH).

TABLE 1 | Distribution of various types of somatic mutations.

Gene	Type of mutation	BRCA1, $n = 78$	BRCA2, $n = 21$	IARC *TP53* database Unknown BRCA, $n = 1249$
TP53	Missense	47	11	
	frameshift deletions	11	4	
	Nonsense	13	2	
	other	4	3	
TP53	GOF*	19 (25%)	3 (15%)	271 (22%)
	LOF*	24 (32%)**	6 (30%)**	261 (21%)**
	Unclassified	32 (42%)	11 (55%)	717 (57%)
	Wild type	3	1	
APC	nonsense	-	1	
BAX	frameshift	1	-	
KRAS	missense (pathogenic)	1	-	
CDKN2A	**missense**			
	unknown	3	-	
	pathogenic	1	-	
CTNNB1	missense (pathogenic)	2	-	

**The percentage of mutations of various types is determined only for cases with mutant TP53.*

***The percentage of LOF mutations in BRCA1/2-deficient tumors is significantly higher than in the reference group not stratified by BRCA status, $p < 0.01$.*

To this end, for each mutation, the variant allele frequency (VAF) was calculated as the ratio of the variant allele reads number to the total number of reads. The tumor content estimated by histological sections assay was used to normalize VAF as :

$$Normalized\ VAF\ =\ \frac{VAF}{\%\ Tumor\ cells}$$

The number of variant allele reads is $(1+K) \times T$, where T is the number of tumor cells in the sample, and k is the proportion of cells with a LOH, so that

$$Normalized\ VAF\ =\ \frac{(1+k)}{2}$$

For all HGSOC samples with one exception, normalized VAFs were either 0.50 ± 0.05 (29/94) or 1.00 ± 0.08 (65/94), which corresponds to the proportion of tumor cells with LOH, $k = 0$ or $k = 100\%$ (**Figure 2**). For one sample carrying a single nucleotide deletion in *TP53*, VAF was 1.6, most likely caused by a deviation in the ratio of amplified alleles at the library preparation stage. We found the rate of the tumors with the LOH-positive variant of TP53 to be about 71% (68/95). Most of the nonsense *TP53* variants (14/15, 93%) were LOH-positive. Among other types of variants, the LOH frequency ranged from 60 to 70% (**Figure 1**).

We did not observe intermediate values $0 < k < 1$ for *TP53* variants, while for 3/9 cases in the APC (1), KRAS (1), CDKN2A (1) genes, the normalized VAF was 0.23–0.37, indicating that only subclones of tumor cells carry these somatic variants.

Immunohistochemical Assay of p53

There is a common agreement that both types of abnormal p53 expression (high and low, or absent) are correlated with mutant p53. The expression level is usually determined by the content of p53 positively stained nuclei. In various studies of HGSOC, the cut-off for high p53 expression levels indicating mutation ranged from 50 to 85% (17–19). In the present study, the cut-off was 70%, as described by Cole et al. (20). We carried out p53 immunohistochemistry for 68/99 patients, with 56 cases (82.3%) found to have abnormal p53 expression (**Table 2** and **Supplementary Table 1**). In addition, we searched for differences in p53 protein levels between patients with GOF, LOF, and "unclassified" variants. Most of the analyzed samples with GOF variants (11/14) were p53 high-positive, while all analyzed tumors carrying LOF variants (9) were low-positive or negative. For the "unclassified" mutants, abnormal p53 expression was found in 31 of 40 cases only. The rest of the "unclassified" tumors had normal (intermediate) p53 expression with the actual number of nuclei staining positive for p53 in the "intermediate" category ranging from 30 to 56% (**Supplementary Table 1**). For the samples with wild-type *TP53,* only one had normal expression (65% of positive nuclei), while three showed low expression or absence of p53.

DISCUSSION

The frequency and spectrum of *TP53* variants are highly variable and depend on the type of cancer (2). It is not yet clear what spectrum of *TP53* variants is specific for *BRCA1/2* associated HGSOC. In our study, we first examined the frequency of various types of *TP53* variants in HGSOC patients with a germline *BRCA1* or *BRCA2* pathogenic variants.

Recently, conflicting evidence has emerged on the association of mutated *TP53* type with platinum treatment resistance (21–24). To exclude the possible influence of this factor, only chemo-naive and chemo-sensitive patients with PFI > 6 months were taken in this study.

Our results showed that the frequency of true LOF variants leading to the truncated protein among *BRCA1/BRCA2* carriers with HGSOC was significantly higher than in reference cohort with HGSOC not stratified by BRCA status and chemo-sensitivity (30.3 vs. 21.0%, $p = 0.01$). This finding overlaps with the results of the study by Dumay et al. (25), in which basal-like breast tumors displayed significantly more truncating variants than luminal tumors (43 vs. 25%). Breast cancer with *BRCA1* (but not *BRCA2*) mutations is known to typically have a basal phenotype. Apparently, there is a causal relationship between an increase in the frequency of truncating variants and *BRCA1* alteration.

Truncating variants usually result in loss of any activity of wild-type p53. In contrast, functional outcomes of *TP53* missense variants can be very diverse: LOF, acquisition of oncomorphic function (GOF), or no effect. According to IARC annotation, in our study, the majority (93%) of the missense mutants lacked transactivation activity (26), whereas GOF was described

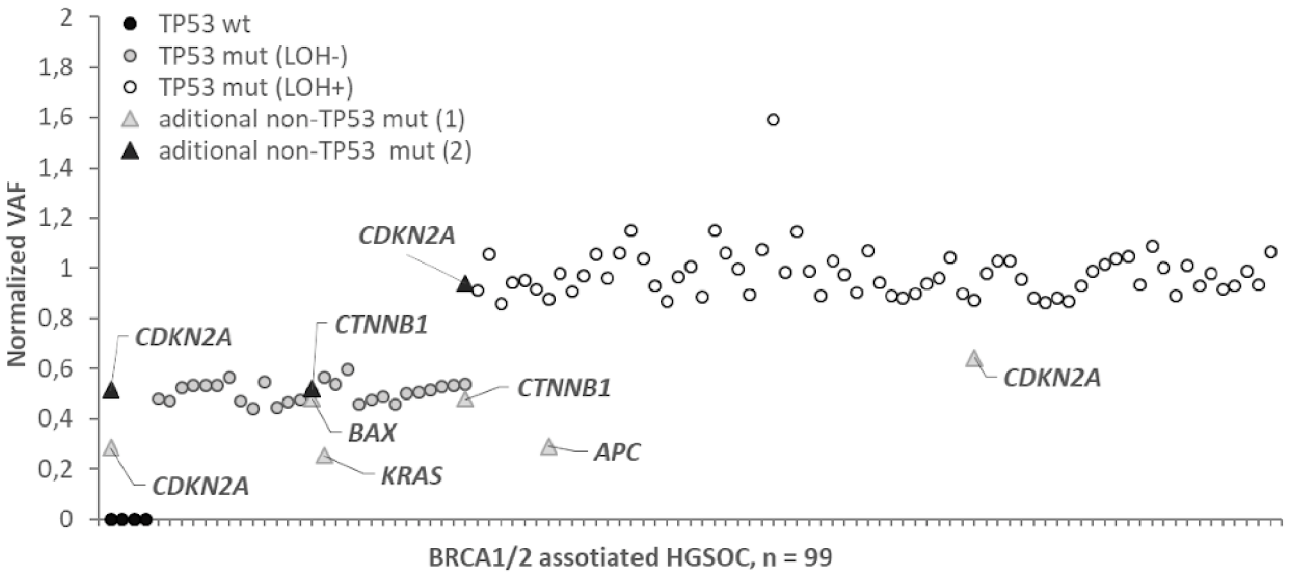

FIGURE 2 | Normalized variant allele frequency (normalized VAF) for somatic mutation in *BRCA1/2* associated HGSOC. Normalized VAFs of 0.5 or 1 correspond to a percentage of LOH positive tumor cells of 0 or 100%. Normalized VAFs of <0.5 indicate that the mutation is contained in the subclones of the tumor; the LOH status for these mutations is not determinable.

for 28 mutants (22, with conventional GOF variants and 6, with "unclassified" variants) (16). However, it should be kept in mind that most experimental studies of GOF were focused on testing the frequent *TP53* variants clustered at codons 175, 245, 248, 249, 273, and 282. For non-hot spot *TP53* variants, there are insufficient data on their GOF properties, making it impossible to perform a system analysis of GOF for all *TP53* variants.

We used the expression level of the TP53 gene as an indirect marker of mutant p53 with GOF properties, as reviewed by other authors (27, 28). It is conceivable that mutant p53 accumulation in tumors is crucial to exert its GOF in carcinogenesis (29–32). To characterize *TP53* variants with respect to p53 protein expression, we performed IHC with 68 samples. Most of the analyzed samples with GOF variants (11/14) were p53 high-positive, while all analyzed tumors carrying LOF variants (9) were p53 low-positive or negative (**Table 2**). These results suggest the absence or low level of expression to predict a loss of p53 function, with a sensitivity of 100% and a specificity of 73%. According to this criterion, 25 of 40 IHC tested "unclassified" missense variants with the low and absent level of p53 expression can be defined as probable LOF. Importantly, only 6/40 "unclassified" mutants were p53 high-positive, and the rest (9) had an intermediate level of protein expression. If an elevated expression of mutant p53 is a factor of pro-oncogenic activity, then 9 variants in the samples with a normal level of p53 expression cannot be classified as GOF.

Four BRCA-deficient HGSOC (4/99) did not have somatic variants in *TP53,* but, surprisingly, 3/4 "normal" samples did show abnormal (low or absent) p53 expression levels, i.e., three of four tumors lost p53 function. A possible reason for the loss of p53 function is the deregulation of p53 stability, for example, through the amplification of *MDM2*, the protein of which regulates p53 proteasome degradation (33).

TABLE 2 | Immunohistochemical classification p53 status for tumor samples with various types of somatic *TP53* mutations.

	Type of *TP53* mutation			
p53 staining	**GOF**	**LOF**	**Unclassified**	**Wild Type**
Negative	4	8	22	2
Positive				
High	11	-	6	-
Intermediate	-	-	9	1
Low	-	1	3	1

An important feature of p53 function is the integrity of the second *TP53* allele. Most often, inactivation of the second allele occurs through copy-neutral LOH. In our study, LOH was determined based on the normalized VAF. Interestingly, for all LOH-negative and LOH-positive samples, the proportion of tumor cells with LOH was close to 0 and 100%, respectively (with one exception). Based on these findings, we hypothesized that there are no significant sub-clonal populations of cells with different LOH-status of *TP53* in primary *BRCA1/2*-associated HGSOC. This assumption is consistent with the driver role of p53 in carcinogenesis. Thus, potential intratumor heterogeneity and clonal evolution under the pressure of treatment or metastasis will result from the selection of concomitant non-*TP53* somatic variants like *BRCA1/2* and other genes.

Since LOH is a sign of driver variant, it can be assumed that the proportion of LOH-positive variants of *TP53* will be close to 100%. It was the case for tumors with nonsense TP53 variants, where the incidence of LOH was 93% (14/15). In other cases, the rate of LOH-positive variants was about 67%. The relatively low frequency of LOH can be explained by the existence of an alternative way to disable the second *TP53* allele through the interference of p53 missense mutants with wild-type p53 (dominant-negative effect, DNE) (34).

In addition to *TP53*, we sequenced the loci of genes containing frequent somatic variants specific to solid tumors, including ovarian cancer. Additional somatic variants were found in six patients. A total of 9 variants were found in the genes *APC* (1), *BAX* (1), *KRAS* (1), *CDKN2A* (4), *CTNNB1* (2). Three out of the six patients (including one patient with normal p53) had variants in the *CDKN2A* gene whose protein products p14ARF and p16INK4a act as tumor suppressors due to negative regulation of the cell cycle (35).

Variants of *BAX*, coding core regulators of apoptosis and (or) *APC* and *CTNNB1* were detected in three tumors. The products of both genes (*APC* and *CTNNB1*) are components of the Wnt-signaling pathway, an important element in the regulation of embryogenesis and cell differentiation. (36). Studies of cancer genetics showed that genes encoding proteins of the Wnt pathway are frequent targets for mutational alterations in various cancers, including colon, prostate, breast, and ovarian cancer (37). The aberrant activation of the APC/b-catenin pathway is suggested to be restricted to endometrioid ovarian cancer. It is possible that these two samples were incorrectly classified as adenocarcinoma or contain subclones with different histological differentiation.

Variants in other classical oncogenes specific for solid tumors were not detected except for the only specimen carrying the *KRAS* variant. Based on the normalized VAFs of *TP53* and *KRAS* variants (0.51 and 0.23, correspondingly), two equally possible clonal architecture of the tumor can be suggested. First, the tumor contains clonal driver mutation of *TP53* (without LOH) and subclonal (affecting about 50% of cells) mutation *KRAS* likely to have occurred later in tumor evolution. Second, mutations of *TP53* (with LOH) and *KRAS* are independent subclones (each affecting about 50%). Perhaps the tumor is a mix of high and low-grade serous cancer (since the primary KRAS mutations are characteristic of low-grade ovarian carcinoma). Then, the question arises of the multiclonal origin of the tumor. According to the current concept of serous carcinoma pathogenesis, the second option seems less likely. However, it should not be excluded.

According to the latest data, the same variants of *TP53* exert different properties depending on the origin, stage, and molecular profile of a tumor. It was previously shown that patients with HGSOC carrying concurrent somatic variants in *TP53* and additional driver oncogenes had a worsened prognostic signature (reduced PFI, time to recurrence and OS) (18). On the other hand, it is known that the p53 mutants cooperate synergistically with other oncogenes (RAS, TGF-b), causing a more aggressive cancer (38–42). In our study, most samples of HGSOC did not have additional (other than *TP53* and *BRCA1/2)* variants in oncogenes, which seems to be a favorable prognostic factor. Apparently, under such microenvironment, potential GOF (pro-oncogenic) properties are less likely to manifest.

This study has potential limitations. (1) As a reference group, the set of variants specific for HGSOC from the IARC TP53 database was used. For most cases of the reference group, ethnicity and BRCA status were not defined. It is likely that some reference cases of HGSOC are associated with BRCA. A case-control study (BRCA1/2 associated vs. sporadic HGSOC) has more sensitivity to detect differences in the compared samples (would be more preferable). The source of bias is the probable ethnicity heterogeneity of the compared samples and, consequently, the heterogeneous structure of inherited genetic factors, for example, prevalence and spectrum of *BRCA1/2* mutations. Therefore, our findings are supposed to be confirmed by studies with large samples adjusted to ethnicity. (2) To determine non-TP53 somatic mutations, a target panel was used that covers the loci most frequently mutated in solid cancers. However, relatively rare cancer genes have not been sequenced although it can be expected that some of them might have clinical relevance.

CONCLUSIONS

We have focused on molecular profiling of chemo-naive and platinum-sensitive HGSOC with germline *BRCA1/2* variants. Using NGS we have analyzed both the set of *TP53* variants and somatic variants of other genes involved in carcinogenesis. Our findings showed that somatic TP53 variant or inhibition of wild-type p53 expression was observed in almost 100% of cases with *BRCA1/2* associated HGSOC. Rare exceptions are accompanied by variants in other genes of the cell cycle, confirming earlier observations that the negative regulation of cell cycle checkpoints is the main hallmark of BRCA-deficient class of HGSOC. With missense variants predominating among *TP53*, the proportion of truncating variants is significantly higher than with a mixed (in terms of BRCA mutations and sensitivity to platinum) cohort of HGSOC. LOH is typical for *TP53* nonsense variants only, while for other types of variants, there is no pattern in the distribution of LOH. Loss of transcription activities is a common property of missense p53 mutants. There are several indirect signs (normal or low expression of mutants p53, low incidence of concomitant oncogenes mutations) indicating the low manifestation of the GOF properties of *TP53* variants in BRCA1/2 associated HGSOC. Due to the driver role of *TP53*, its variants will persist in all tumorous subclones during treatment or metastasis. This knowledge can be useful in the management of patients with advanced ovarian cancer.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/sra/PRJNA612603, Accession no: PRJNA612603.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institute of Molecular Biology and Biophysics Ethics Committee (Protocol: # 1 dated March 14, 2017). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

UB and LG: conceptualization. UB: funding acquisition. DL, NK, and AMA: resources (patients and clinical description). EK, AMA, and NK: investigation. AK, AM, and AA: software. UB and AK: formal analysis. MF: supervision. UB and LG: writing—original draft. All authors: writing—review and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.01103/full#supplementary-material

Supplementary Table 1 | List of *BRCA1/2* germline mutations and somatic mutations in the HGSOC samples.

Supplementary Table 2 | List of the custom NGS panel loci.

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MINI REVIEW
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Key Players in the Mutant p53 Team: Small Molecules, Gene Editing, Immunotherapy

Vitaly Chasov[1], Regina Mirgayazova[1], Ekaterina Zmievskaya[1], Raniya Khadiullina[1], Aygul Valiullina[1], Joseph Stephenson Clarke[2], Albert Rizvanov[1], Matthias G. J. Baud[2] and Emil Bulatov[1,3]**

[1] Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Russia, [2] School of Chemistry, University of Southampton, Southampton, United Kingdom, [3] Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia

The transcription factor p53 is a key tumor suppressor that is inactivated in almost all cancers due to either point mutations in the *TP53* gene or overexpression of its negative regulators. The p53 protein is known as the "cellular gatekeeper" for its roles in facilitating DNA repair, cell cycle arrest or apoptosis upon DNA damage. Most p53 mutations are missense and result in either structural destabilization of the protein, causing its partial unfolding and deactivation under physiological conditions, or impairment of its DNA-binding properties. Tumor cells with p53 mutations are generally more immunogenic due to "hot spot" neoantigens that instigate the immune system response. In this review, we discuss the key therapeutic strategies targeting mutant p53 tumors, including classical approaches based on small molecule intervention and emerging technologies such as gene editing and T cell immunotherapy.

Keywords: p53, mutation, small molecules, adenoviral gene therapy, CRISPR/Cas gene editing, immunotherapy

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***Correspondence:**
Matthias G. J. Baud
m.baud@soton.ac.uk
Emil Bulatov
chembio.kazan@gmail.com

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INTRODUCTION

The transcription factor p53 functions as a tumor suppressor and is considered as one of the most promising molecular targets for cancer therapy, as it regulates a plethora of intracellular metabolic pathways, including DNA damage repair, apoptosis, and senescence. The p53 protein is widely known as the "guardian of the genome" that prevents the proliferation of cells harboring genetic aberrations, notably oncogenic mutations. In both stressed and unstressed cells, the p53 protein is subject to post-translational modifications, including phosphorylation, acetylation, ubiquitination, and methylation that regulate its stability, localization (cytoplasm or nucleus) and transcriptional activity. Phosphorylation of Ser or Thr residues of p53 was shown to correlate with increasing of p53 activity in response to cellular stress (1).

The *TP53* gene encoding the p53 protein is the most frequently altered gene in human tumors (2). The loss of transcriptional functions leading to the deactivation of intrinsic tumor suppressive responses associated with wild-type (WT) p53 is the primary outcome of p53 mutations, and is a hallmark of most cancers The majority of p53 mutations are missense, i.e., cause single residue substitutions, and occur within the DNA-binding domain (DBD). These can be classified as either "DNA contact" or "conformational" mutations (3). "DNA contact" mutations occur in regions that make direct contact with target DNA sequences and are critical for DNA binding, whereas "conformational" mutations diminish DNA-binding by distorting the protein structure through destabilization. Most of these mutations are loss-of-function

and exert a dominant negative effect on the WT protein functions. Beyond this, cancer cells appear to gain selective advantages by retaining only the mutant form of the protein, associated with enhanced cell proliferation, metastasis and chemoresistance (4).

The intracellular p53 level is tightly regulated by its negative regulator murine double minute 2 (MDM2) ubiquitin ligase, mostly through ubiquitination followed by proteasomal degradation. In most human cancers, p53 is deactivated either due to mutation or because of the overexpression of MDM2. The strategy of enhancing p53 functions by means of small molecule MDM2 inhibitors has long been of interest to the field by its perceived tractability (5). However, despite development of dozens of high-affinity compounds and multiple clinical trials, none have yet produced a registered drug, suggesting that alternative paths should be given greater attention (6). The MDM2-induced degradation of p53 could be regulated by p14ARF that inhibits the oncogenic action of MDM2 and enhances p53-dependent transactivation and apoptosis (7).

The general approaches employed to destroy the p53-mutant tumor cells are implemented either *via* restoration of its WT oncosuppressor properties, or focus on tumor elimination by manipulating key components of the immune system. In this review we discuss the current and emerging therapeutic strategies against mutant-p53-driven cancers based on small molecule re-activators, gene editing technologies (introduction of WT gene or CRISPR/Cas mediated corrections) and T cell immunotherapy (**Figure 1**).

DEFENSIVE STRATEGY: SMALL MOLECULE RE-ACTIVATORS

MDM2 is mostly known for its oncogenic properties, though its role beyond cancer, notably inflammation, has received increasing attention in recent years (8–10). Numerous synthetic modulators that activate WT p53 by MDM2-dependent, e.g., Nutlin-3a (11–13), and MDM2-independent mechanisms (14–16) have been reported. However, Nutlins and similar inhibitors of MDM2 often demonstrated side effects in clinical trials, such as off-target issues and dose-limiting hematological toxicities, e.g., thrombocytopenia and neutropenia.

Chemoresistant MDM2 mutations were also reported to evolve, although there is evidence that this may be addressed by combination therapies using stapled-peptide MDM2 antagonists. Such mutations occur in *N*-terminal p53-binding domain, zinc finger and RING domains. "Stapled" peptide inhibitor (PM2) has been reported, which has a covalent hydrocarbon linkage bridging the adjacent turns of an alpha helical peptide for improved stability (17). The peptide recapitulated key p53 signature residues and targeted the *N*-terminal domain of MDM2. The structural mimicry and extended spatial contacts with the protein allowed PM2 to retain binding ($K_D = 117$ nM) to mutant forms of MDM2 resistant to Nutlins.

Targeting tumors with mutant *TP53*, both somatic and germline, presents a challenging yet potentially highly rewarding approach as such mutations are the main driver of various types of cancer (18). The equilibrium between the properly folded and misfolded states of p53 can be affected by compounds that interact with mutant p53 and reinstate its native fold and function (**Figure 2B**). A number of small molecules have been developed to target and stabilize specific mutant forms of p53 and restore WT resembling transcriptional activity, thereby leading to cell cycle arrest or apoptosis of mutant tumor cells. While many tumor suppressor genes are predominantly inactivated in cancer through deletion, truncating mutations or epigenetic mechanisms, the majority of p53 cancer mutations are missense mutations which lead to the expression of functionally altered full-length mutant p53 proteins with single amino acid substitutions. Approximately one third of oncogenic p53 mutants are conformationally unstable due to specific "hot spot" residues that are mutated at a disproportionately high frequency, most of which reside in the structured p53 DNA-binding region (19). The nine most frequent mutations (R175H, R248Q, R273H, R248W, R273C, R282W, G245S, R249S, Y220C), the majority of which are DNA contact mutants, account for about 30% of all p53 cancer driving mutations.

Such "contact" mutants not only lose their transcriptional activity due to impaired DNA binding, but also exhibit dominant-negative (DN) effects on the remaining WT p53 allele in addition to the homologous tumor-suppressors p63 and p73 (20). Mutant p53 proteins can form heterotetramers with WT p53, hampering the function of the latter in tumor suppression (21). The primary outcome of *TP53* mutations leading to loss of WT p53 functions is the abrogation of its intrinsic tumor suppressive responses such as senescence and apoptosis, while gain-of-function mutant p53 proteins enhance tumor progression, metastatic potential, and drug resistance, greatly contributing to the malignant cellular phenotype (22–24).

Most p53 mutants lose their transcriptional activity and tumor suppressive function, although approximately a third of p53 mutants are temperature sensitive and display sequence-specific transcriptional activity at sub-physiological temperatures (25, 26). Interestingly, introduction of rationally designed second-site suppressor mutations was shown to stabilize the structure of the p53 DBD and reactivate transcription, providing access to valuable WT like variants for screening and drug discovery (27, 28). At the same time, this suggests that stabilization of such "conformational" mutants may provide an opportunity to reinstate their WT function through the use of modulators of their thermal stability. There is currently enormous interest in the identification of natural or synthetic substances (small molecules, peptides, etc.) that can stabilize mutant p53 in its

Abbreviations: ACT, adoptive cell therapy; APC, antigen-presenting cells; BE, base editor; CAR, chimeric antigen receptor; CFDA, China Food and Drug Administration; CRISPR/Cas, clustered regularly interspaced short palindromic repeats/CRISPR-associated; DBD, DNA-binding domain; DC, dendritic cell; DSB, double strand break; ECM, extracellular matrix; HDR, homology directed repair; MDM2, murine double minute 2; MDS, mutant myelodysplastic syndrome; MHC, major histocompatibility complex; MQ, methylene quinuclidinone; nCas9, Cas9 nickase; PBLs, peripheral blood lymphocytes; PE, prime editor; pegRNA, prime editing guide RNA; ROS, reactive oxygen species; TALEN, transcription activator-like effector nuclease; TCR, T cell receptor; TILs, tumor-infiltrating lymphocytes; TMB, tumor mutational burden; TME, tumor microenvironment; TLR, Toll-like receptor; WT, wild-type; ZFN, zing-finger nuclease; ZMC, zinc metallochaperone.

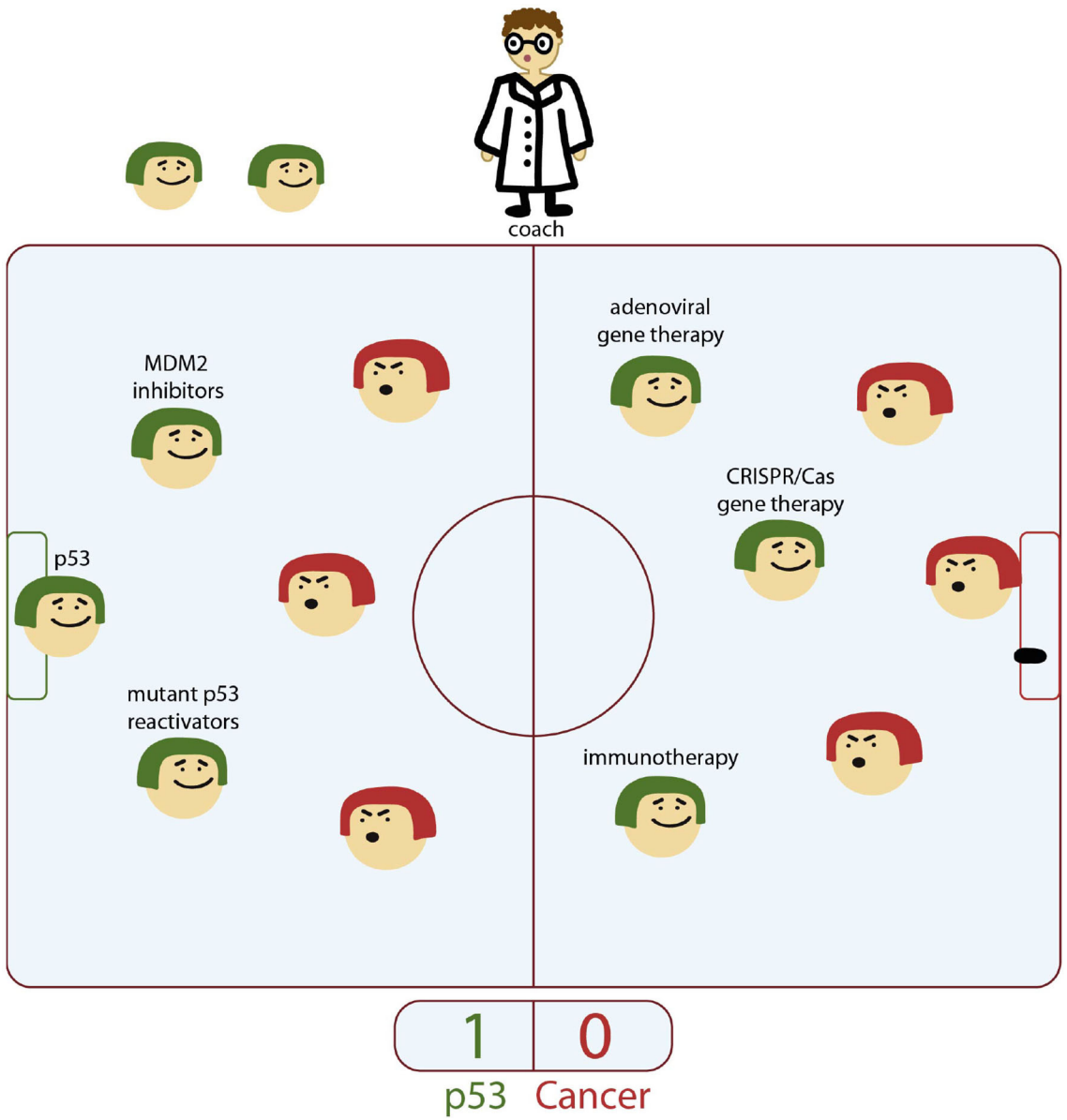

FIGURE 1 | Key players in the p53 team. P53 is the genome "gatekeeper." MDM2 inhibitors, mutant p53 re-activators are the players of defensive line, whereas adenoviral gene therapy, gene editing tools, and immunotherapy are part of the offensive line in p53 team. New and yet unknown powerful players are expected to enter the game at the forefront of cancer treatment and score a success under the researcher coaching.

active biological conformation and restore DNA-binding and transcriptional activity (29).

PRIMA-1 and its methyl analog APR-246 (PRIMA-1^{MET}) are promising small molecules that can restore activity of mutant p53 by interacting with the DNA binding domain, promoting proper folding/function (29). This leads to enhanced expression of pro-apoptotic genes *Puma*, *Noxa,* and *Bax* in p53 mutant cells in addition to activation of cell-cycle genes and PARP cleavage independent of p53 mutation status, as observed in multiple studies that involved various types of cancer such as breast, thyroid, myeloma (30).

Both PRIMA and APR-246 are pro-drugs that are intracellularly converted to the reactive methylene quinuclidinone (MQ), which covalently binds to surface-exposed cysteine residues of mutant p53 as well as WT p53. At the same time, experiments with recombinantly expressed and intracellular p53 proteins have shown that unfolded mutant p53 was modified by PRIMA-1 more efficiently than the correctly folded WT protein (31). MQ may also exert its anticancer effect *via* an alternative p53-independent mechanism of action based on glutathione (GSH) depletion, leading to upregulation of reactive oxygen species (ROS) levels and modulation of

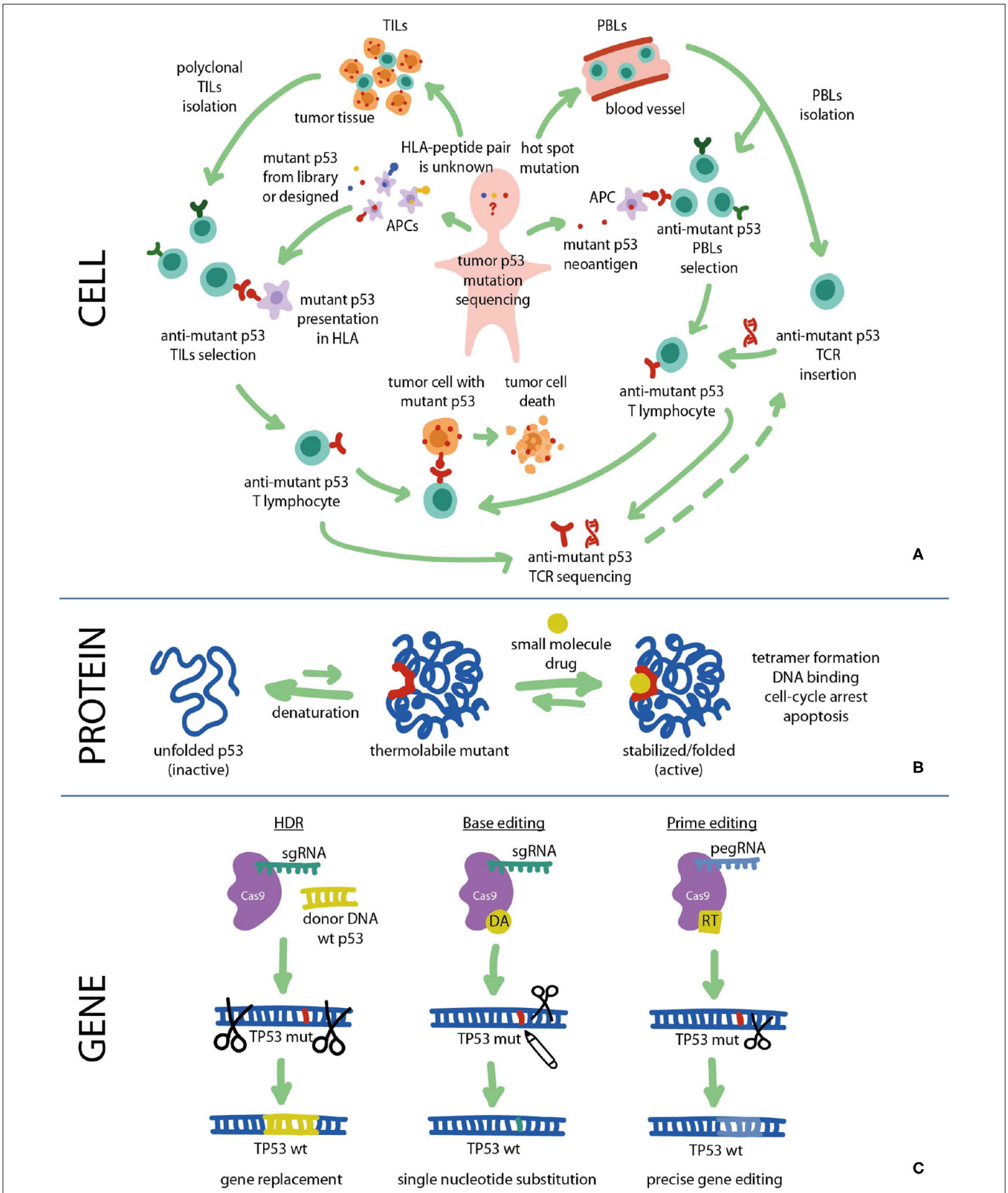

FIGURE 2 | Fighting cancer via p53 pathway can be implemented at all levels: cell, protein, and gene. **(A)** Cancer cells carrying mutant p53 can be targeted with immunotherapy using mutant p53-specific TILs or TCR-T cells. **(B)** At the protein level the DNA-binding and transcriptional functions of mutant p53 can be restored using small molecule re-activators that stabilize the protein in its active biological conformation. **(C)** At the gene level *TP53* mutations can be repaired using CRISPR/Cas9 gene editing approaches such as HDR, Base editing and Prime editing. HDR, homology directed repair; HLA, human leukocyte antigen; PBL, peripheral blood lymphocyte; RT, reverse transcriptase; TCR, T cell receptor; TIL, tumor-infiltrating lymphocyte.

the intracellular redox state (32). Currently, APR-246 in combination with azacitidine has reached Phase III clinical trial for the treatment of *TP53* mutant myelodysplastic syndromes (MDS) (NCT03745716) and Phase II for *TP53* mutant myeloid neoplasms (NCT03072043, NCT03588078).

Bauer et al. (33) identified a range of 2-sulfonylpyrimidines as mild arylating agents of surface cysteines in both WT p53 and mutant p53 core domains. Cysteine arylation upon treatment with lead molecule PK11007 stabilized the mutant p53 core domain *in vitro* by up to 3°C in differential scanning fluorimetry experiments. In cells, it induced concentration-dependent upregulation of several p53 target genes (*p21*, *PUMA*) in cancer cell lines, although p53-independent cytotoxicity was also observed in p53-null and WT p53 cell lines. Interestingly, PK11007 also induced strong GSH depletion and ROS upregulation in cells, reminiscent of the cellular profile and suggested mode of action of MQ and its derivatives. Altogether, these studies highlighted the important effect of cellular redox modulation and a potential general strategy for the development of covalent anticancer agents targeting mutant p53 and redox pathways synergistically, although the propensity for off-target redoxive cell damage by such agents is high.

The Y220C mutation is the ninth most frequent p53 missense mutant overall and is associated with over 100,000 new cancer cases per year worldwide, predominantly breast and ovarian cancer (18, 34). Behind the most common "contact" mutations (*vide infra*), it is by far the most frequent "conformational" p53 cancer mutation. This large-to-small residue mutation creates an extended cavity on the protein surface that destabilizes the DBD by ∼4 kcal/mol (35), causing denaturation and aggregation. The hydrophobic and "druggable" nature of the Y220C pocket offers a fruitful opportunity for targeting using small-molecule stabilizers. Critically, the mutation-induced crevice is distant from the p53 surfaces involved in DNA recognition or protein-protein interactions, allowing for the development of targeted chemical agents that stabilize the DBD without interfering with binding of its natural substrates.

In recent years, fragment-based and *in silico* screening methods have led to the identification of several potent lead compound families that bind the Y220C pocket. A range of carbazole derivatives displaying low micromolar affinity increased the melting temperature of p53-Y220C and slowed its rate of aggregation *in vitro*. PK9328 ($K_D = 2\,\mu M$) induced cell viability reduction of several Y220C cancer cell lines, although some toxicity was also observed in other cell lines not carrying this mutation, possibly suggesting off-target effects (36). Pyrazole derivative PK7088 rescued the folding of p53-Y220C and restored transactivation and downstream upregulation of *p21* and *Noxa* expression, correlating with cell cycle arrest and apoptosis (37).

Recently, our group reported several potent iodophenol lead molecules displaying low micromolar binding affinity *in vitro*, thermal stabilization of up to 2.2°C and selective pro-apoptotic activity in a panel of Y220C cancer cells. Structure-activity studies culminated in aminobenzothiazole derivatives MB710 and MB725, which demonstrated *in vitro* K_D up to 4 μM for p53-Y220C by isothermal titration calorimetry (38). MB725 also showed potent and selective viability reduction of several p53-Y220C cancer cell lines such as BXPC-3 (pancreatic adenocarcinoma), HUH-7 (hepatocellular carcinoma), NUGC3 (gastric adenocarcinoma), while maintaining comparatively low toxicity in WT p53 WI38 (normal fibroblasts), and NUGC4 (gastric adenocarcinoma) in the same concentration range. Importantly, the correlation between *in vitro* thermal stabilization and selective viability reduction in Y220C cell lines represents an important milestone toward first-in-class anticancer drugs that rescue p53-Y220C function. This provides a compelling rationale for future lead optimization efforts toward potent, non-toxic targeted agents for reactivating the Y220C mutant in anticancer therapy.

ZMC-1 (zinc metallochaperone-1) is a thiosemicarbazone-based small molecule that rescues the WT protein folding and transcriptional activity of p53-R175H mutant by buffering the intracellular Zn^{2+} levels (39). The underlying rationale is that zinc is required for the correct folding of WT p53 protein and mutations that impair zinc binding strength can hamper protein stability and conformation, leading to impaired sequence-specific DNA binding to p53 response elements (3, 40). ZMC-1 restored site-specific DNA binding and upregulation of p53 target genes (*p21*, *Puma*, *Mdm2*) (41), and inhibited mouse xenograft tumor growth with high allele-specificity for the p53-R175H (p53-R172 in mice) mutant. While zinc buffering alone was insufficient to induce apoptosis (41), ZMC-1 also activated p53 by induction of ROS through its ability to chelate other metal ions (Fe^{2+}, Fe^{3+}, Cu^{2+}) (42). The 3rd-generation thiosemicarbazone COTI-2 functions similarly through both p53-mediated pathways and p53-independent redox homeostatic mechanisms (43) and has entered a Phase II clinical trial (NCT02433626), although it is of note that thiosemicarbazone cancer drug candidates have known nonspecific cytotoxicity and effects on iron metabolic pathways (44).

OFFENSIVE STRATEGY: GENE THERAPY AND IMMUNOTHERAPY

Adenoviral Gene Therapy

Gene therapy is a promising therapeutic option and some practical examples have already been studied and successfully applied to re-establish WT p53 expression and activity in cancer cells. Gene therapy involves the replacement or addition of a correct copy of the abnormal gene with a view to restore the genetic information, thus reinstating the WT phenotype.

Currently, gene therapy approaches are based on the combination of genetic material with suitable delivery systems that are often limited by the requirement for efficient nuclear delivery and gene expression. Several primary delivery systems for *TP53* gene-based therapeutics have been developed using various viral vectors, including adenoviral, retroviral, vaccine-derived vectors and non-viral ones based on liposomes, polymeric, and gold nanoparticles that allow overcoming systemic delivery hurdles (45). Currently, adenoviral vectors demonstrate minimum side effects among viral vectors used for *TP53* gene therapy.

Up until now, several clinical studies using viral vectors for the delivery of p53 have been conducted for experimental medicines, such as Advexin and Gendicine. Advexin (Introgen Therapeutics Inc., TX, USA) is an adenoviral-based experimental therapeutic that provided delivery of WT p53 to cancer cells and demonstrated anticancer activity following amended expression of p53 (46). Gendicine, based on recombinant human p53 adenovirus (Shenzhen SiBiono GeneTech Co. Ltd., China), was approved in 2003 by the China Food and Drug Administration (CFDA) as a first-in-class gene therapy product to treat head and neck cancer, and entered the commercial market in 2004 (47).

Novel adenoviral vectors for cancer gene therapy targeting the p53 pathway were developed to improve the transgene expression levels. Two adenoviral vectors were reported that differ only in the promoter site: the constitutive CMV promoter and the p53-responsive PG promoter where a p53-responsive element is inserted in the viral vector (48). The p53 expression was found to be substantially higher in PCa cells after transduction with AdPGp53 compared to AdCMVp53, and DU145 cells were particularly susceptible to the AdPGp53 tumor suppressor properties.

However, the application of viral vectors can induce high immunogenicity and enhance pre-existing immunity, which limits their clinical use and requires development of new systems with equal efficiency but better safety profiles. Non-viral vectors could present significant advantages when compared with viral ones due to their safety and low cost; nevertheless, viral vectors currently dominate gene therapy clinical trials because of their relatively high delivery efficiency. Thus, viral vectors for the delivery of WT *TP53* gene are seen as strong players in the p53 team, however, introduction of other powerful players would increase the firepower of the offensive line.

CRISPR/Cas Gene Editing

There are numerous molecular tools for programmable genome editing at a clinical level, including zinc-finger nucleases (ZFNs) (49, 50), transcription activator-like effector nucleases (TALENs) (51, 52), clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) (53). CRISPR/Cas is widely seen as a revolutionary technology for biomedical research with immense clinical opportunities for treating cancer and genetic disorders.

In 2016 the laboratory of David Liu at Harvard University developed an advanced version of CRISPR/Cas enzymes, called Base Editors (BEs), which can mediate specific point mutations in genomic DNA and the resulting amino acid sequence of a target protein (54, 55). BEs constitute enzymatically inactive Cas9 nickase (nCas9) fused to either cytidine deaminase (cytidine BE) or adenosine deaminase (adenosine BE) that result in cytosine-to-thymine or adenine-to-guanine conversion in DNA. In human cells BEs function with high efficiency (15–75%) and low indel rates (<0.1%) compared to classical CRISPR/Cas9 technique based on homology directed repair (HDR). BEs could significantly advance treatment of mutation-associated cancer and genetic diseases by specifically correcting pathogenic mutations in the target gene.

In 2019 the same laboratory reported new gene editing tool, Prime Editors (PEs), based on even more advanced CRISPR/Cas9 "search-and-replace" technology (56). Here, the desired genetic information is directly introduced using nCas9 fused to reverse transcriptase that is directed by prime editing guide RNA (pegRNA) specifying the target DNA sequence and encoding the genetic edits. PEs expand the list of available genome editing tools and together with BEs they can potentially correct ∼89% of all known pathogenic human genetic variants.

Several clinical trials are in progress to apply CRISPR/Cas9 for the treatment of patients with mutation-associated disorders, such as β-thalassemia (NCT03655678, NCT03728322) and sickle cell disease (NCT03745287) whereby genetic manipulations with blood cells are carried out *ex vivo* and then gene-corrected cells are infused back to the patient. A particularly remarkable example is Leber congenital amaurosis 10 (NCT03872479), for which CRISPR-based investigational therapy is administered *in vivo* via subretinal injection.

Oncogenic or disease-causing mutations represent the primary targets for gene editing therapies. The highest mutation rate of *TP53* among other genes makes it a highly desirable target for gene editing tools, e.g., to reverse missense mutation back to the WT state. Chira et al. (57) proposed a CRISPR-based delivery system of a functional *TP53* gene. According to the authors, the entire mutated *TP53* locus could be deleted and then replaced with a functional copy by homologous recombination. In principle, this might be feasible because the CRISPR/Cas9 system is capable of making such large insertions (58). As a result, the WT phenotype of *TP53* could be recovered by replacing the perturbed gene with its functional copy leading to normal p53 expression and tumor regression.

CRIPSR/Cas9 gene editing, including Base Editing, Prime Editing and upcoming technologies have set a high expectations bar for future clinical applications (**Figure 2C**). BEs, PEs and similar approaches that allow introduction of precise genetic corrections into a target locus without deleting the whole gene could potentially be used to correct *TP53* missense mutations as a prospective anticancer therapy (59). Given the rapid advancement of CRISPR/Cas9 technologies and their inevitable introduction to clinical practice, both *ex vivo* and *in vivo* target gene modifications in a wide range of cancers, including solid tumors, does not seem to be a distant future anymore.

However, efficient intracellular delivery remains one of the main barriers on the path for wider clinical application of CRISPR/Cas9 technology, including for the purposes of therapeutic editing of *TP53* gene. There are three primary strategies for intracellular delivery of CRISPR/Cas9 components: viral vectors, lipid nanoparticles and Cas9-sgRNA complexes. Among these the viral gene delivery strategy seems to be the closest to clinical practice because it has been used in classical gene therapy for decades (60). CRISPR/Cas9-induced double strand breaks (DSBs) of the genomic DNA can result in cell cycle arrest or cell death through p53 pathway that induces DNA damage response and activates expression of downstream effector proteins, e.g., cell cycle inhibitor $p21^{CIP1/WAF1}$. Functioning of

the cellular DNA repair mechanisms that get activated upon DSB, which is often an integral initial step of the gene-editing mechanism, explains one of the reasons for low efficiency of the classical CRISPR/Cas9 system (61, 62).

The rapid development of CRISPR/Cas9-based technologies for therapeutic gene editing of the *TP53*-associated pathologies is expected to enhance precision, enable improved correction of point mutations, provide better delivery, reduce side effects and facilitate wider clinical applications.

Immunotherapy

TP53 mutations as part of the overall tumor mutational burden (TMB) can be considered an important factor in predicting response to immunotherapy. *TP53* missense mutation-associated p53 nuclear accumulation results in a higher local density of tumor-infiltrating lymphocytes (TILs) within the primary tumor (63). The p53 protein can regulate the immune landscape by modulating inflammation, senescence and immunity in the surrounding tumor microenvironment (TME), including tumor stroma, extracellular matrix (ECM) and associated immune cells infiltrate (64). Mutation in p53 can lead to enhanced neo-angiogenesis and ECM remodeling, disruption of innate tumor immunity, genotoxic stress response of the Toll-like receptor (TLR) pathway, favor pro-tumor macrophage signature and alter cell-mediated immunity in cancer (65).

Some pathways leading to T cell exhaustion are upregulated in such tumors, therefore making them a good target for immunotherapeutic treatment based on genetically modified T cells, e.g., T cell receptor (TCR)-T cells or chimeric antigen receptor (CAR)-T cells (66).

Tumor cells elicit immunogenic responses due to "hot spot" mutant p53 epitopes (neoantigens) produced *via* proteasomal degradation of intracellular protein and presented by major histocompatibility complex (MHC) (**Figure 2A**). Initial studies showed that tumors with mutated *TP53* could be recognized by peripheral blood lymphocytes (PBLs) upon *in vitro* stimulation and *in vivo* immunization (67–69). Cancer vaccines based on primed autologous dendritic cells (DCs) reactive to neoepitopes lead to enhanced antitumor T cell responses in ovarian cancer patients and were associated with better survival prognosis (70).

Tumor-specific adoptive cell therapy (ACT) using antigen-experienced T cells, e.g., patient's own autologous TILs, is a novel approach for targeting p53 mutant cancers. In this approach a HLA/neoantigen complex is recognized by T cell receptors (TCRs) of cytotoxic T cells that effect tumor lysis. Particularly interesting are genetically-engineered T cell receptor (TCR)-T cells with known HLA/neoantigen combination generated by transduction or transposition of specific TCRs into autologous or allogeneic T cells (71). Limitations of this method include differentiation status and proliferative potential of TILs/TCR-Ts, and most importantly potential loss of HLA on tumor cells that would restrict the efficiency of T cell-mediated cytotoxicity.

Deniger et al. (72) prospectively evaluated intratumoral T cell responses to autologous somatic mutant p53 neoantigens expressed by human metastatic ovarian cancers. T cells with specificity to mutated neoantigens found in high frequencies in TILs were expanded from resected metastases and then co-cultured with autologous antigen-presenting cells (APCs) expressing mutated p53 epitopes (Y220C and G245S). Immunogenicity of T cell response was confirmed by upregulation of 4-1BB or secretion of IFNg.

Lo et al. (73) screened TILs for recognition of mutated neoantigens in metastatic colorectal cancer patients and observed T cell mediated recognition of immunogenic p53-R175H mutant. Several TCRs were also identified that could be transduced into allogeneic PBLs for ACT application as an off-the-shelf TCR-T cell product targeting cancer cell lines with a wide range of *TP53* mutations.

Malekzadeh et al. (74) developed a *TP53*-specific screening assay to evaluate T cell responses to "hot spot" mutant p53 neoantigens introduced to autologous APCs intracellularly (tandem minigenes) or extracellularly (pulsed peptides). TCRs from CD4+ and CD8+ T cells reactive to mutant p53 neoantigens were identified in lung cancer patients and then TCR-T cells were engineered that recognize the same HLA/neoantigen complex. In follow-up experiments they isolated PBLs from patients with mutant p53 (R175H, Y220C, R248W) tumors by sorting antigen-experienced CD4+ and CD8+ T cells (75). The T cells were then stimulated with p53 neoantigens (naturally occurring processed and presented peptides) *in vitro* to confirm the recognition and specificity of the immune response.

Future studies will reveal detailed mechanisms of the complex regulatory interplay between the tumor *TP53* status and the immune landscape, including p53-mediated innate anti-tumor response and presentation of mutant p53 neoantigens for eliciting immune recognition by T cell receptors.

CONCLUSION

The set of available molecular tools arming scientists to battle somatic mutation-associated tumors and hereditary diseases has expanded significantly in recent years. Traditional approaches such as rational structure- and fragment-based drug discovery targeting protein interfaces have been successfully complemented with innovative gene- and cell-based technologies. Adenoviral gene therapy and CRISPR/Cas gene editing are advancing in clinical trials for the treatment of mutation-linked diseases, and the expansion of their applications for therapeutic targeting of *TP53* mutations inevitably also approaches. Immunotherapy based on genetically engineered T cells (either autologous or allogeneic) complement cancer treatment by providing unique specificity and efficiency. Therefore, the key players in the mutant p53 team—small molecules, adenoviruses, CRISPR/Cas gene editing enzymes, T cell-based therapies and combinations thereof—broaden the therapeutic scope and provide enormous clinical potential for targeting p53 mutant tumors at all levels (gene, protein and cell). We believe that these approaches have truly encouraging opportunities for clinical applications and that major advancements based on them are approaching in the near future. Together they will fuel challenging, but highly rewarding new developments in the field of mutant p53 cancer therapy.

AUTHOR CONTRIBUTIONS

VC, JS, and MB contributed to section about small molecules. RM and RK contributed to section about gene editing. AV and EZ contributed to section about immunotherapy. EZ and RM prepared the figures. VC, AR, MB, and EB contributed to introduction and conclusion. VC, MB, and EB conceived the idea and coordinated the writing. All authors contributed to the article and approved the submitted version.

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p53 Affects PGC1α Stability Through AKT/GSK-3β to Enhance Cisplatin Sensitivity in Non-Small Cell Lung Cancer

*Xinyue Deng[1], Yang Li[1], Shuang Gu[2], Yingying Chen[1], Bingbing Yu[1], Jing Su[1], Liankun Sun[1] and Yanan Liu[1]**

[1] Key Laboratory of Pathobiology, Ministry of Education, Department of Pathophysiology, College of Basic Medical Sciences, Jilin University, Changchun, China, [2] Department of Thoracic Surgery, Jilin Provincial People's Hospital, Changchun, China

Drug resistance greatly limits the therapeutic efficacy of treatment of non-small cell lung cancer (NSCLC). One of the important factors is the dysfunction of tumor suppressor p53. Recent studies have suggested that p53 suppresses tumors by regulating number of mitochondrial proteins, including peroxisome proliferator-activated receptor coactivator (PGC1α). Although several studies have confirmed the interaction between p53 and PGC1α, the precise mechanism has not been completely determined in NSCLC. In this study, we investigated the specific signaling between p53 and PGC1α to improve anti-tumor drug effects on NSCLC. We found that low expression of p53 and high expression of PGC1α correlated with shorter survival time of NSCLC patients. *In vitro* experiments confirmed that NCI-H1299 (p53-null) cells had high levels of PGC1α and were insensitive to cisplatin (CDDP). When PGC1α was knocked down, the sensitivity to cisplatin was increased. Notably, the stability of PGC1α is an important mechanism in its activity regulation. We demonstrated that p53 decreased the stability of PGC1α via the ubiquitin proteasome pathway, which was mediated by protein kinase B (AKT) inhibition and glycogen synthase kinase (GSK-3β) activation. Therefore, p53 may regulate the stability of PGC1α through the AKT/GSK-3β pathway, thus affect the chemosensitivity of NSCLC.

Keywords: non-small cell lung cancer, p53, PGC1α, mitochondrial function, AKT, GSK-3β, CDDP

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***Correspondence:**
Yanan Liu
ynliu@jlu.edu.cn

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BACKGROUND

Lung cancer is one of the most malignant tumors in the world, and the main type is non-small cell lung cancer (NSCLC). In recent years, drug resistance has become a problem in the treatment of NSCLC, leading to poor prognosis (1). Tumor suppressor p53, a major defense factor against cancer, initiates apoptosis by triggering a caspase cascade (2). However, about half of the NSCLC subtypes have p53 missense mutations, resulting in loss of wild type p53 activity (3, 4). Tumors with loss of p53 function are often resistant to chemotherapy drugs (5–7). Therefore, for NSCLC patients with p53 dysfunction, seeking new targeted treatment has become the key to overcoming chemoresistance.

p53 regulates many cellular functions, including cell cycle arrest, senescence and apoptosis, to inhibit tumorigenesis (8). Recent studies have found that it is also involved in the regulation of

tumor suppression though other functions, such as metabolic reprogramming, and antioxidant, and mitochondrial function regulation. An increasing number of mitochondrial proteins involved in mitochondrial metabolism and respiration are regulated by p53 (9, 10). Peroxisome proliferator-activated receptor coactivator (PGC1α), a master regulator of mitochondrial function, mediates mitochondrial biogenesis, oxidative phosphorylation, and mitochondrial reactive oxygen species (ROS) detoxification. Several studies have confirmed the interaction between p53 and PGC1α (11), however, its regulatory mechanism has not been completely determined. Aquilano et al. have found that p53 binds to the promoter region of *PPARGC1A* to induce its expression, and depletion of the antioxidant factor, glutathione, induces the p53-PGC1α-Nuclear factor 2 (NRF2) axis (12). However, Villeneuve et al. have demonstrated that p53 inhibits PGC1α and induces oxidative stress in cardiomyocytes (13). Additionally, PGC1α plays an important role in tumor chemotherapy drug resistance. Upregulation of PGC1α protected tumor cells from cisplatin (CDDP) cytotoxicity by regulating mitochondrial respiratory chain complex proteins and the oxygen consumption rate (OCR) in colon cancer (14). Gao et al. have also found that targeting PGC1α reduced the drug resistance of melanoma to mitogen-activated protein kinase (MAPK) inhibitors (15). Taken together, we speculated that the regulatory relationship between p53 and PGC1α is an important drug resistance mechanism of NSCLC.

Owing to the short half-life of PGC1α, its stability regulation is an important mechanism of its activity regulation (16). PGC1α activity is modulated by both expression and posttranscription modifications. Rozalyn et al. have found that PGC1α degradation by the proteasome system depends on glycogen synthase kinase (GSK-3β)-mediated phosphorylation (17). Additionally, the serine/threonine-specific kinase, Akt, plays an important role in a variety of cellular processes. After activation, Akt is transferred to different subcellular compartments to phosphorylate the multifunctional serine/threonine-specific kinase, GSK-3β, to inhibit its activity (18). Previous studies have found that p53 inhibits the proliferation and metastasis of osteosarcoma by inhibiting the PI3K/AKT/mTOR pathway (19). Rueda-Rincon et al. have also confirmed that p53 affects cell survival by inhibiting the oncogenic AKT pathway (20). Thus, we speculated that p53 affects the stability of PGC1α through the AKT/GSK-3β pathway.

Here, we investigated whether p53 regulates the stability of PGC1α through the AKT/GSK-3β pathway, and thus affects the chemosensitivity of tumor cells. Our results showed that low p53 expression and high PGC1α expression correlated with poor survival rate. Furthermore, p53 affected mitochondrial biosynthesis by regulating PGC1α to reduce chemoresistance of NSCLC. Moreover, our results indicated that PGC1α may be a potential target for individualized treatment of patients with different p53 backgrounds.

Abbreviations: NSCLC, non-small cell lung cancer; PGC1α, peroxisome proliferator-activated receptor coactivator; CDDP, Cisplatin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AKT, protein kinase B; GSK-3β, glycogen synthase kinase; RTCA, real-time cell analysis.

METHODS AND MATERIALS

Reagents and Antibodies

The human non-small lung cancer cell lines, A549, H1975, and H1299, were obtained from the cell bank of the Institute of Biochemistry and Cell Biology (Shanghai, China). A549 cells were cultured in F-12K medium, and H1975 and H1299 cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA). CDDP, RIPA and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St Louis, MO, USA). MG132, Epoxomicin (Epox), cycloheximide (CHX), and GSK-3β inhibitor (CHIR99021) were purchased from MedChemExpress (Monmouth Junction, NJ, USA). Transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Anti-PGC1α(M), anti-p53(M), anti-p21(M), and anti-Nrf1(M) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Bcl-2 (R), anti-Mcl-1(R), anti-Bax (R) and anti-phospho-Akt (phospho T315/316/312) were from Abcam (Cambridge, MA, USA). Anti-cleaved caspase-3 (R), anti-GSK-3β (R), anti-phospho-GSK-3β (phospho Ser9, R), and anti-AKT (R) antibodies were from Cell Signaling Technology (Danvers, MA, USA). Anti-ubiquitin (R) and anti-actin (M) antibodies were from Proteintech (Chicago, IL, USA).

Non-Small Lung Cancer Tissue Microarray and Immunohistochemistry

Tissue microarrays of 90 lung cancer tumors and their corresponding adjacent non-cancer tissues were obtained from Shanghai Outdo Biotech Co., Ltd. (Shanghai, China). Immunohistochemical (IHC) staining was carried out on 5-μm-thick sections of the abovementioned tissues to assess PGC1α and p53 expression. DAPI was used to stain nuclei. Images were acquired using an Aperio slide scanner and analyzed by ImageScope software (Aperio, Shanghai Outdo Biotech, China). For IHC scoring, the percentage (0, 0%; 1, 1–25%; 2, 26–50%; 3, 51–75%; and 4, >75%) of stained tumor cells was multiplied by the intensity (0, 1, 2, or 3) to achieve a score between 0 and 12.

Cell Viability Assay

Cells (8,000 cells per well) were seeded in 96-well-plates and transfected with a PGC1α-shRNA plasmid and/or treated with CDDP for 24 h. MTT reagent was added and cells were incubated for 4 h. Formazan crystals were dissolved in 150 μL of dimethyl sulfoxide and the optical density at 570 nm was recorded by an enzyme-linked immunosorbent assay reader after the plate was shaken for 5 min.

ATP Production

Cells were lysed with a lysis buffer, and then centrifuged (10,000 × g for 2 min) at 4°C. The level of ATP production was determined by mixing 10 μL of the supernatant with 100 μL of luciferase reagent (ATP Bioluminescence Assay Kit, Beyotime Technology, Shanghai, China). The emitted light was measured using an Omega luminometer (BMG Labtech, Ortenberg, Germany). Measurements were normalized to the protein concentration.

Plasmids and Transfections

A full-length human p53 expression vector was constructed by subcloning a full-length *p53* cDNA fragment into pcDNA3.1 vector (Genechem, Shanghai, China). shRNA sequences targeting human *PGC1α* and a non-target sequence were constructed by Genechem. The PGC1α shRNA sequences used were: PGC1α shRNA 1: 5′-GTT-ATA-CCT-GTG-ATG-CTT-T-3′; PGC1α shRNA 2: 5′-CAG-CGA-AGA-TGA-AAG-TGA-T-3′; PGC1α shRNA 3: 5′-AGA-GTA-TGA-CGA-TGG-TAT-T-3′; and the non-target shRNA (Scramble) sequence was 5′-TTC-TCC-GAA-CGT-GTC-ACG-T-3′. Taking 6-well-plate as an example, the amount of plasmid is 4 μg/per well. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Western Blotting

Whole-cell lysates were prepared and quantified according to standard protocols. Lysates diluted with 5 × SDS-PAGE loading buffer were boiled at 95°C for 10 min and separated by SDS-PAGE, and then electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% milk followed by successive incubation with primary antibodies and peroxidase-conjugated secondary antibodies. The bands were visualized using Pierce ECL Western Blot Substrate (Thermo Scientific, Waltham, MA, USA).

RT-PCR and qRT-PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. The reverse transcription reaction and PCR were performed using the SuperScript RT-PCR kit (Thermo Scientific). The target DNA fragments were amplified with their corresponding primers: *ACTB*: 5′-ATATCGCGTCGCTGGTCGTC-3′ (forward) and 5′-AGGATGGCGTGAGGGAGAGC-3′ (reverse); *PPARGC1A*: 5′-CAGAGAGTATGAGAAGCGAGAG-3′ (forward) and 5′-AGCATCACAGGTATAACGGTAG-3′ (reverse). The amplified products were either detected by PCR or separated by 2% agarose gel and detected using ultraviolet light. qRT-PCR was performed using the MX3000P instrument (Agilent, USA).

Real-Time Cell Analysis (RTCA)

The cell growth status was monitored by the RTCA S16 System (ACEA Biosciences, San Diego, CA, USA), as previously reported (21).

Detection of Protein Half-Life

For the protein half-life assay, cells were treated with 200 μM CHX (MedChemExpress) after transfection with p53 and collected at different time points. Then, cells were lysed for western blot analysis.

Co-immunoprecipitation

Cells were lysed with NP40 lysis buffer plus protease inhibitors. Equal amounts of protein lysates were incubated with the indicated antibodies overnight at 4°C (2 μg antibody per 300–500 μg protein), followed by incubation with 30 μL of protein A/G agarose beads (Beyotime Biotechnology). The next day, the beads were rinsed three times with PBS, resuspended in 5 × SDS-PAGE loading buffer, boiled at 95°C for 10 min and centrifuged. The proteins in the supernatant were analyzed by western blot analysis.

Flow Cytometry

Cells were seeded in 6-well-plates and treated with various reagents as indicated. Cells were then harvested and stained with Annexin V-FITC and propidium iodide (PI) (Annexin V Apoptosis Detection Kit, BD Pharmingen, San Jose, CA, USA) to measure cellular apoptosis. The mitochondrial membrane potential (MMP) was determined using the Mitochondrial Membrane Potential Assay Kit (Beyotime Biotechnology). ROS production was evaluated by DCFH-DA (Beyotime Biotechnology). Analysis was performed using a BD Accuri C6 flow cytometer (BD Bioscience) or a BD FACSAria II (BD Bioscience). Data analysis was performed using FlowJo v10 or BD Accuri C6 Software.

Fluorescence Microscopy

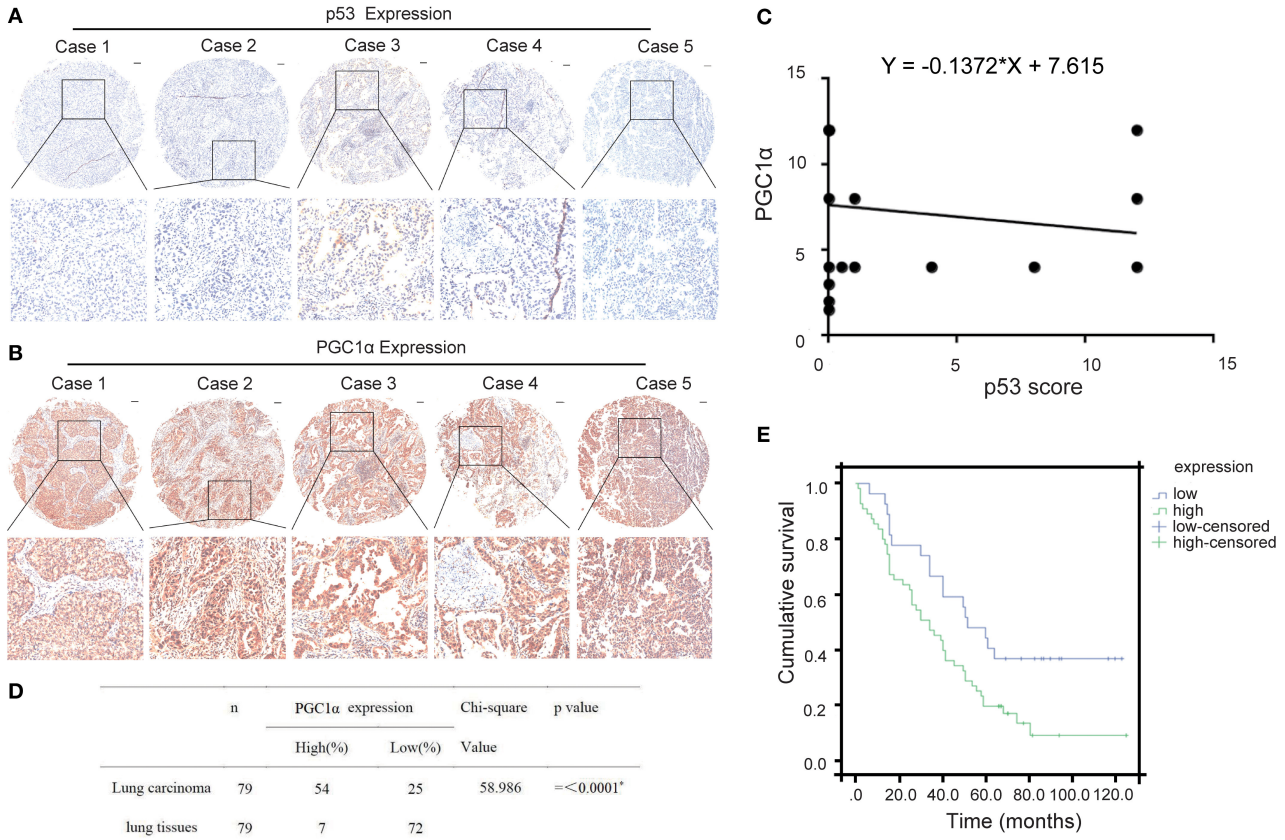
Cells were seeded on glass cover slips in a 24-well-plate and treated as indicated. Then, cells were washed with PBS, fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 for 8 min. After blocking with 5% bovine serum albumin (BSA) for 30 min, cells were incubated with primary antibody overnight at 4°C. After PBS washing, the cells were incubated at room temperature for 1 h in the dark with FITC/Texas Red-conjugated secondary antibodies (Proteintech). The images were observed on an Echo-lab Revolve microscope (CA, USA).

In vivo Xenograft Experiments

Animal experiments were performed following the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Animal Welfare and Ethics Group of the Laboratory Animal Science Department, Jilin University (Changchun, China). H1299 cells (3×10^6) were subcutaneously injected into the upper flank of 4-week-old female BALB/C nude mice purchased from the Beijing Vital River Laboratory Animal Technology (Beijing, China). Two weeks after the injection, the mice were randomly divided into four groups (four mice per group): control, CDDP+Scr-shRNA, PGC1α-shRNA, and CDDP+PGC1α-shRNA. CDDP (3 mg/kg) was intraperitoneally administered every 2 days and 100 μL of PGC1a-shRNA plasmid formulated with attenuated Salmonella Typhi strain Ty21a (1×10^7 CFU/100 L) were injected every week. The body weight and tumor volume were recorded every 2 days. After 21 days of treatment, mice were sacrificed and tumors were dissected, weighed, and photographed.

Tunel Assay

Mouse tumor tissues were fixed in 4% (w/v) paraformaldehyde, dehydrated in ethanol gradient, and embedded in paraffin. Samples were then cut into 3-μm sections using a Leica microtome. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was carried out according to the manufacturer's instructions (Roche Ltd., Mannheim Germany). Sections were analyzed



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	n	PGC1α expression		Chi-square	p value
		High(%)	Low(%)	Value	
Lung carcinoma	79	54	25	58.986	=<0.0001*
lung tissues	79	7	72		

*statically significance (p <0.05)

FIGURE 1 | Expression of PGC1α and p53 in human non-small lung cancer tissues. **(A,B)** Immunohistochemical staining of PGC1α and p53 in NSCLC tissue microarrays (40×). Typical staining is shown in the adjacent rows (100×). **(C)** PGC1α expression negtively corrected with p53 expression in NSCLC tissues analyzed by spearman correlation (Rho = −0.341, $P < 0.01$). **(D)** Expression of PGC1α in lung carcinoma and lung tissues ($P = 0.00$). **(E)** Kaplan–Meier survival curve shows significant correlation between high PGC1α expression and low survival in human NSCLC.

using an inverted fluorescence microscope (Olympus, Tokyo, Japan).

Statistical Analysis

Data are expressed as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered statistically significant. Statistical analysis was performed with GraphPad Prism 5 (La Jolla, CA, USA). All experiments were repeated at least three times.

RESULT

The Expression of p53 and PGC1α Correlates With the Survival Rate of Non-Small Cell Lung Cancer

Previous studies have reported that p53 binds to the promoter region of *PGC1α* and regulates its activity (22). To determine the relationship between p53 and PGC1α, we investigated the expression of p53 and PGC1α in human NSCLC tissues from 90 patients by immunohistochemical staining. Spearman correlation analysis showed that the p53 expression negatively correlated with PGC1α expression (Rho = −0.341, $P < 0.01$; **Figures 1A–C**). Furthermore, the tumor tissues had increased PGC1α expression compared with the normal lung tissues (**Figure 1D**), and the increased expression of PGC1α was associated with low survival rate of NSCLC as assessed by Kaplan–Meier analysis ($P = 0.017$; **Figure 1E**). Taken together, these results suggest that there is negative relationship between p53 and PGC1α, and that PGC1α may be a potential target for treatment of NSCLC with low p53 expression.

Chemosensitivity to CDDP Is Determined by Both p53 and PGC1α Expression in NSCLC Cells

To further investigate the relationship between p53 and PGC1α in NSCLC cells, we examined the expression of PGC1α in cells with different variants of p53, that is, A549 (p53 wild type), H1975 (p53 mutant), and H1299 (p53-null). We found that compared with A549 and H1975, H1299

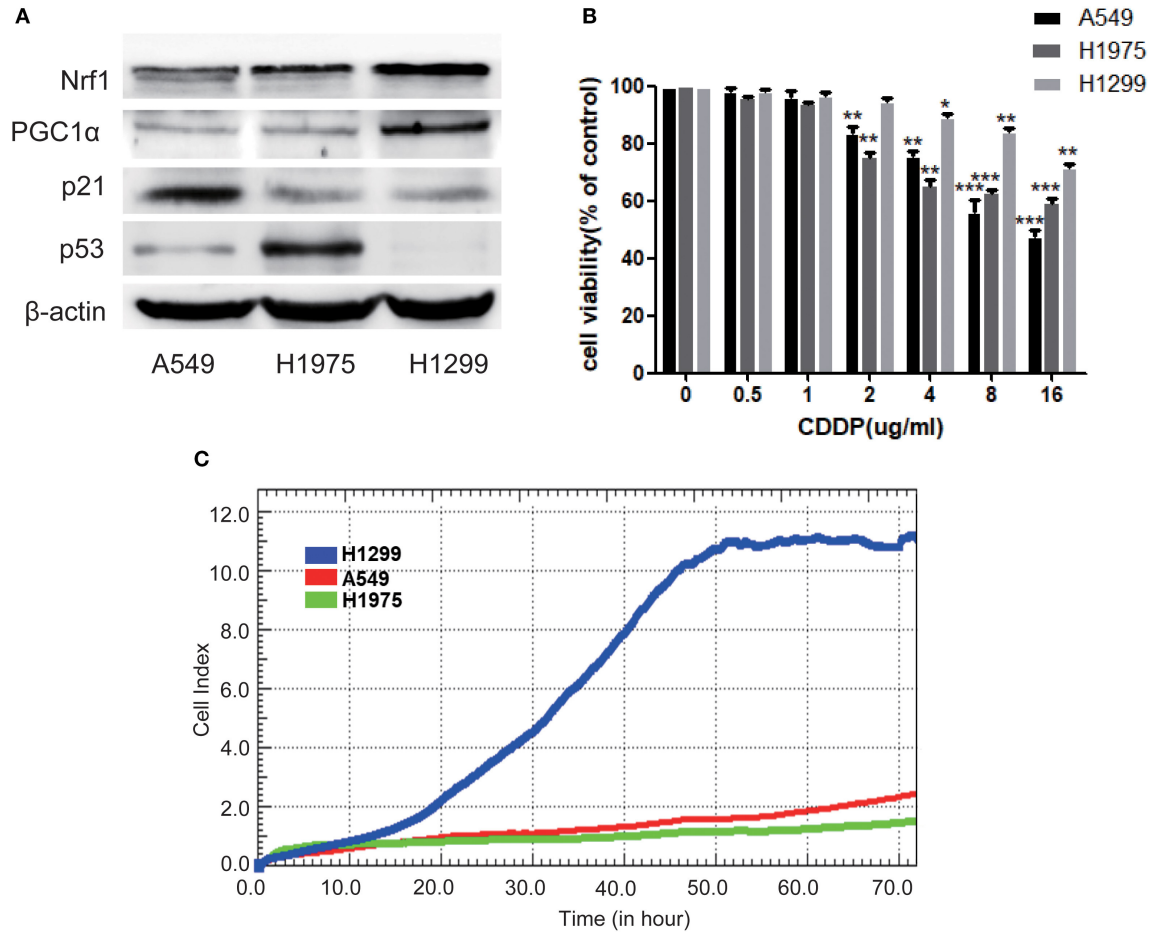

FIGURE 2 | The chemosensitivity to CDDP is determined by both the expression of p53 and PGC1α in NSCLC cells. **(A)** The expression of PGC1α and its downstream target Nrf1, and of p53 and its downstream target p21 in A549 (p53 wild type), H1975 (p53 mutant), and H1299 (p53-null) cells was examined by western blotting. **(B)** A549, H1975, and H1299 cells were treated with different doses of CDDP for 24 h. Cell viability was determined by the MTT assay. Data are the mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the respective controls. **(C)** Time-dependent cell growth curve of human non-small lung cancer cells. The cell suspensions were transferred to E-Plates and placed on the RTCA reader for real-time monitoring every 5 min for the duration of the assay. The number of cells inside the well is displayed as the Cell Index.

had increased expression of PGC1α and its downstream target, Nuclear factor 1 (Nrf1) (**Figure 2A**). Mutations or deletions in the *TP53* gene primarily result in impaired tumor suppressor function (23). Notably, loss of p53 function is linked to resistance to chemotherapeutic agents (24), while increased PGC1α expression leads to drug resistance by upregulating oxidative phosphorylation (OXPHOS) (25). Next, we determined the sensitivity to CDDP using the MTT assay, which revealed that the cell viability of H1299 cells was significantly higher than that of A549 and H1975 cells after CDDP treatment (**Figure 2B**). Furthermore, RTCA indicated a shorter time for H1299 cells to reach the logarithmic phase, compared with the other cells (**Figure 2C**). These results suggest that H1299 (p53-null) cells have high PGC1α expression, which decreases their sensitivity to CDDP treatment.

p53 Affects the Stability of PGC1α Through the Ubiquitin Proteasome Pathway

As shown above, there was a negative correlation between p53 and PGC1α expression. To investigate how p53 regulates PGC1α, we examined the effect of p53 on PGC1α at both the mRNA and protein levels. We first demonstrated that p53 was successfully overexpressed (**Supplementary Figure 1**). The RT-PCR and qRT-PCR results showed that p53 promoted the expression of *PPARGC1A* at the mRNA level (**Figures 3A,B**). However, there was a decrease in the expression of PGC1α protein and the downstream proteins, Nrf1 and Mitochondrial transcription factor A (Tfam), after p53 overexpression (**Figure 3C**). Consistently, the immunofluorescent staining of PGC1α was also decreased (**Figure 4G**). These results prompted us to ask whether p53 affects the stability of PGC1α. Hence, we examined the degradation rate of PGC1α

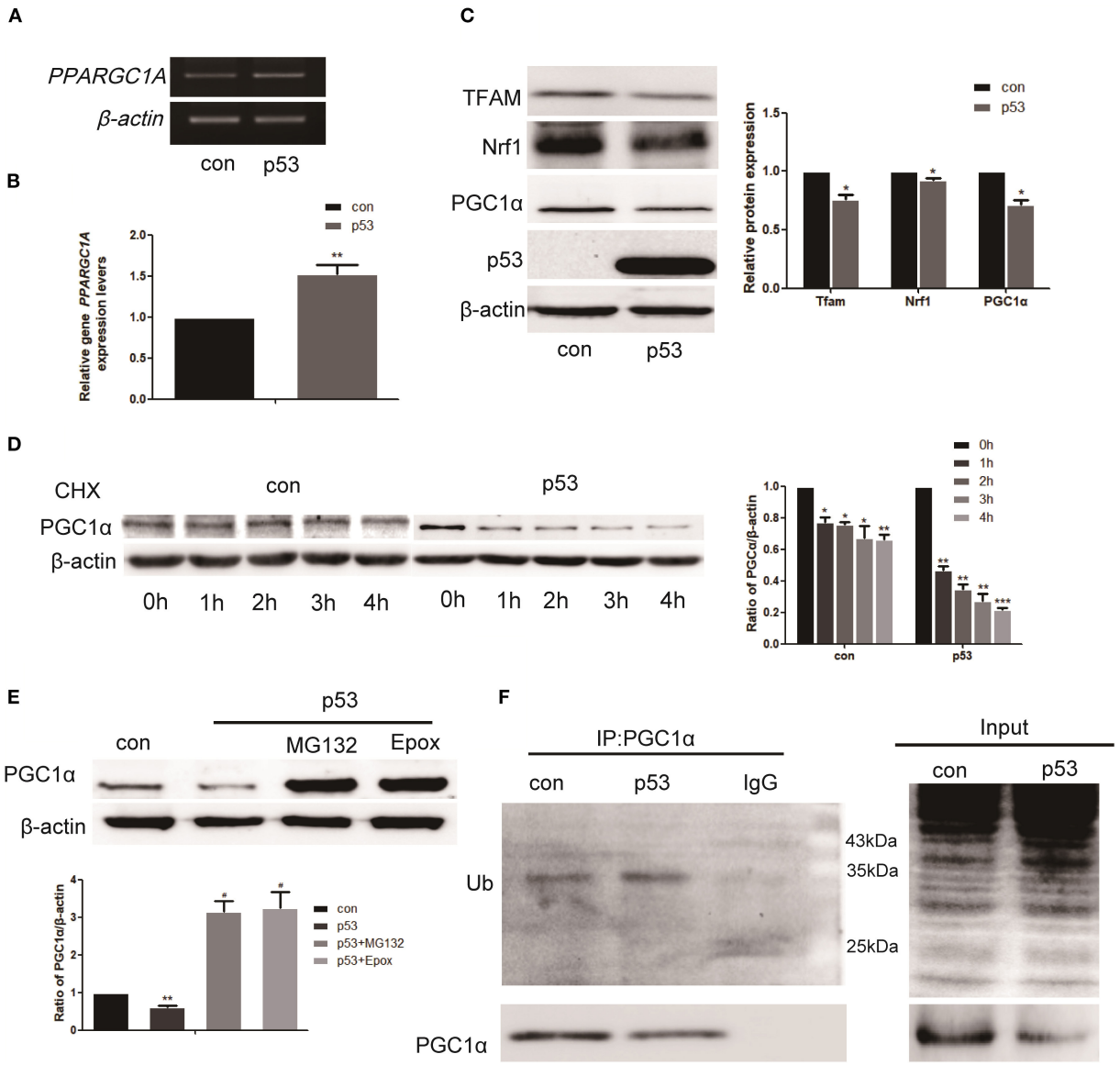

FIGURE 3 | p53 affects the stability of PGC1α through the ubiquitin proteasome pathway. H1299 cells were transfected with a p53 overexpression construct or pcDNA3.1 (empty vector as the control group) for 48 h. **(A,B)** Relative *PGC1α* expression was measured by RT-PCR and qRT-PCR. Data are the mean ± SD, $n = 3$, $^{**}P < 0.01$, compared with the control. **(C)** Western blot analysis of the expression of PGC1α and its downstream targets Tfam and Nrf1. Data are the mean ± SD, $n = 3$, $^{*}P < 0.05$, compared with the control. **(D)** H1299 cells transfected with p53 or empty vector for 24 h were treated with 200 μM CHX, collected at the indicated time points, and analyzed by western blotting. Data are the mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the control. **(E)** H1299 cells were treated with the proteasome inhibitors, 2 μM MG132, and 50 nM Epox, for 18 h after transfection with p53. The expression of PGC1α was examined by western blotting. Data are the mean ± SD, $n = 3$, $^{**}P < 0.01$, compared with the control, $^{\#}P < 0.05$, compared with the p53 group. **(F)** H1299 cells were transfected with p53 or empty vector for 48 h. Immunoprecipitation was performed with anti-PGC1α antibodies followed by western blotting using anti-ubiquitin and anti-PGC1α antibodies.

by translation inhibition experiments using Cycloheximide (CHX), which is widely used for exploring protein degradation (26, 27). The level of PGC1α in p53-overexpressing H1299 cells decreased significantly at 1 h compared with the control group (**Figure 3D**), indicating that p53 decreased the stability of PGC1α.

As the proteasome is one of the principal mechanisms for specific depletion of proteins, we used proteasome inhibitors (MG132 and Epox). Western blot analysis showed that the PGC1α levels increased in the presence of MG132 and Epox (**Figure 3E**). Proteins degraded by the proteasome are polyubiquitinated on their polypeptide chains (28). Therefore, we performed immunoprecipitation of PGC1α to investigate the presence of PGC1α-ubiquitin conjugates in H1299 cells. Western blot analysis demonstrated that the ubiquitinated form was moderately enriched in precipitates from cells

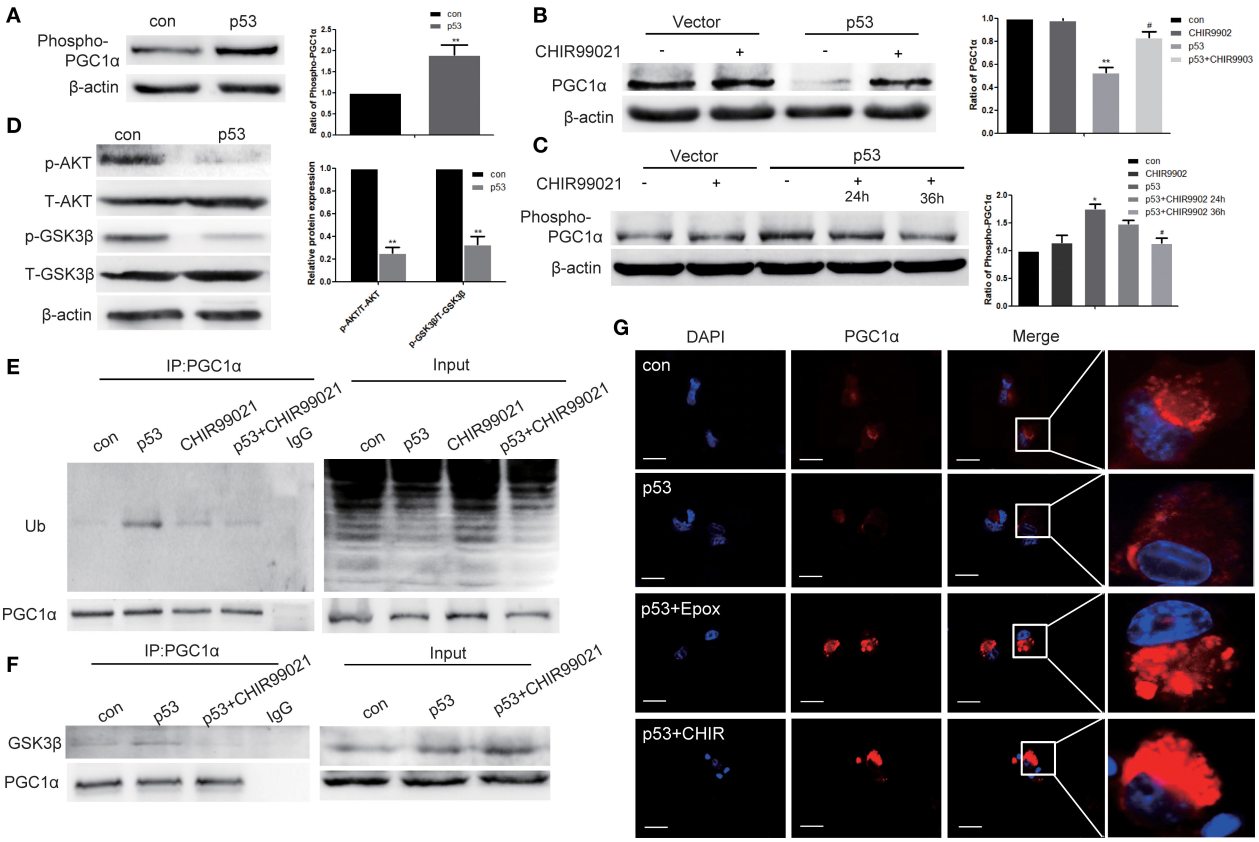

FIGURE 4 | p53 promotes the degradation of PGC1α by the ubiquitin proteasome pathway via AKT/GSK-3β-dependent phosphorylation. H1299 cells were transfected with p53 or empty vector for 48 h. The expression of phospho-PGC1α **(A)** and p-AKT, T-AKT, p-GSK-3β, T-GSK-3β **(D)** were measured by western blotting. Data are the mean ± SD, $n = 3$, $^{**}P < 0.01$, compared with the control. H1299 cells were treated with the GSK-3β inhibitor, 5 μM CHIR99021, for 24 h or 36 h after transfection with p53 or empty vector. **(B,C)** Western blotting determined the expression of PGC1α and phospho-PGC1α. Data are the mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control; $^{\#}P < 0.05$, compared with the p53 group. **(E,F)** Immunoprecipitation was performed with the anti-PGC1α antibody followed by western blotting using anti-ubiquitin, anti-GSK-3β, and anti-PGC1α antibodies. **(G)** H1299 cells were treated with 50 nM Epox for 18 h or 5 μM CHIR99021 for 36 h after transfection with p53 or empty vector. PGC1α expression was determined by immunofluorescence staining (magnification ×400).

overexpressing p53 (**Figure 3F**). These data indicate that p53 decreased the stability of PGC1α by the ubiquitin proteasome pathway.

Degradation of PGC1α by p53 Requires AKT/GSK-3β-Dependent Phosphorylation

Proteins are often phosphorylated before being recognized by the ubiquitin-proteasome pathway. Western blotting showed increased phospho-PGC1α after transfection with p53 (**Figure 4A**). Previous studies have confirmed that GSK-3β-mediated phosphorylation primes BMAL1 for subsequent degradation via proteasomal degradation. We asked whether p53 induced PGC1α degradation through GSK-3β. In the presence of GSK-3β inhibitor (CHIR99021), the decreased levels of PGC1α after transfection with p53 was reversed (**Figure 4B**). Furthermore, the increased expression of phospho-PGC1α decreased after CHIR99021 treatment (**Figure 4C**). GSK-3β activity is regulated by inhibitory phosphorylation and p53 promotes GSK-3β activity by inhibiting AKT. Next, we measured the phosphorylation levels of GSK-3β and AKT after transfection with p53. Western blot analysis revealed that p53 decreased the phosphorylation of both AKT and GSK-3β, indicating that GSK-3β was activated (**Figure 4D**).

To further confirm whether GSK-3β was directly involved in PGC1α-ubiquitin degradation, we conducted immunoprecipitation experiments using cells transfected with p53 in the absence or presence of GSK-3β inhibitor. The results showed that the enhanced PGC1α ubiquitination after transfection with p53 was reversed by GSK-3β inhibitor (**Figure 4E**). Moreover, p53 slightly increased the co-immunoprecipitation of GSK-3β with PGC1α, and this association was impaired in the presence of GSK-3β inhibitor (**Figure 4F**). To further confirm this, we performed immunofluorescence experiments, which showed that

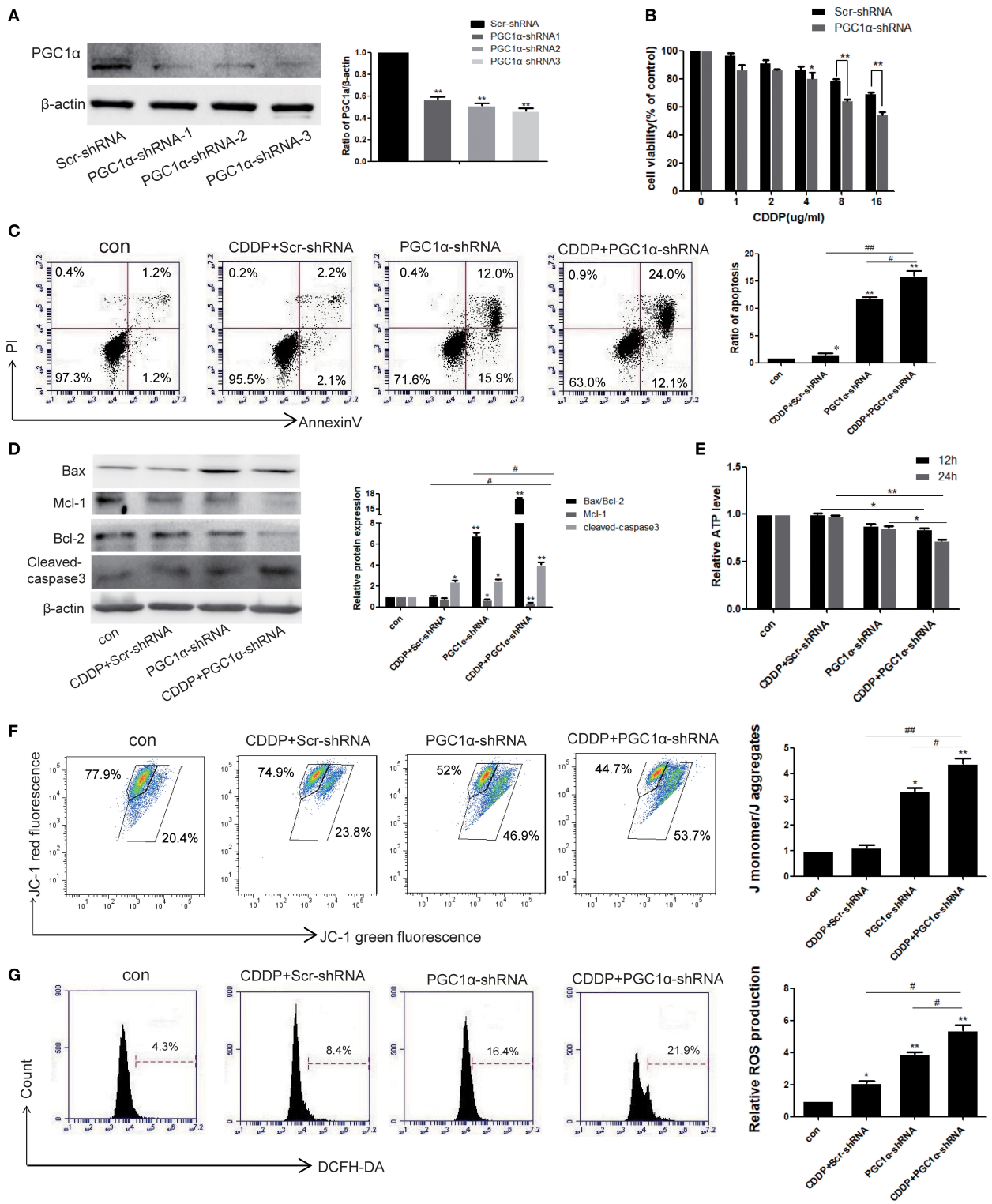

FIGURE 5 | Knockdown of PGC1α combined with CDDP treatment promotes apoptosis by reducing mitochondrial function. **(A)** H1299 cells were transfected with three PGC1α-shRNA plasmids and Scr-shRNA for 48 h. Western blotting was used to analyze the knockdown efficiency. **(B)** Cell viability of transfected H1299 cells exposed to different doses of CDDP for 24 h was determined by the MTT assay. Data are the mean ± SD, $n = 3$, $^{*}P < 0.05$, compared with the control, $^{**}P < 0.01$, compared with the respective CDDP-treated Scr-shRNA group. **(C,D)** H1299 cells were treated with CDDP (8 μg/mL) for 24 h after transfection with PGC1α-shRNA plasmid or Scr-shRNA plasmid. Annexin V/PI staining **(C)** and western blotting analysis of Bax, Bcl2, Mcl-1, and cleaved caspase-3 **(D)** were used to detecte apoptosis. Data are the mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. **(E)** ATP production in the

(Continued)

FIGURE 5 | transfected cells was determined by a kit after treatment with CDDP for 12 or 24 h. Data are the mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$. **(F,G)** Cells transfected with PGC1α-shRNA plasmid or Scr-shRNA plasmid were treated with CDDP (8 μg/mL) for 24 h. Cells were stained with JC-1 **(F)** or DCFH-DA **(G)**, followed by flow cytometry to evaluate the MMP and ROS level. Data are the mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

PGC1α staining was elevated after treatment with Epox or GSK-3β inhibitor combined with p53 overexpression (**Figure 4G**).

PGC1α Knockdown Combined With CDDP Promotes Apoptosis by Reducing Mitochondrial Function

To further verify that high expression of PGC1α is associated with CDDP resistance, PGC1α was knocked down in H1299 cells by transient transfection with shRNA (**Figure 5A**). The MTT assay demonstrated that PGC1α knockdown increased the sensitivity of H1299 cells to CDDP compared with the Scr-shRNA group (**Figure 5B**). Next, after treatment with CDDP and/or transfection with PGC1α-shRNA for 24 h, we examined apoptosis by Annexin V/PI staining and western blotting. The results showed an increase in apoptosis after PGC1α knockdown or CDDP treatment, and the level of apoptosis was further increased in the combined group (**Figure 5C**). Additionally, the expression of the apoptotic proteins, cleaved caspase-3 and Bax, was increased and that of the antiapoptotic proteins, MCl-1 and Bcl-2, was decreased after transfection with PGC1α-shRNA or CDDP treatment, and this effect was further enhanced in the combined group (**Figure 5D**). Furthermore, we measured the ATP level. The results showed that transfection with PGC1α-shRNA or CDDP treatment reduced the ATP content in H1299 cells, and the combined group showed a further decrease (**Figure 5E**). Next, JC-1 fluorescent staining was used to measure MMP. The results showed that transfection with PGC1α-shRNA or CDDP treatment reduced the MMP, which was further decreased in the combined group (**Figure 5F**). We also observed more ROS production in the PGC1α-shRNA and CDDP combined group (**Figure 5G**). These results suggest that PGC1α knockdown combined with CDDP treatment promoted apoptosis by impairing mitochondrial function.

Effects of PGC1α Knockdown Combined With CDDP Treatment on *in vivo* Tumor Xenografts

To examine the effects of PGC1α knockdown and CDDP treatment *in vivo*, we established tumor xenografts by inoculating H1299 NSCLC cells in immunodeficient BALB/C nude mice. We found that PGC1α knockdown improved the effects of CDDP treatment and inhibited tumor growth (**Figures 6A–C**). Western blot analysis showed that the expression of the apoptotic proteins, Bax and cleaved caspase-3, was increased, while the expression of proapoptotic Bcl-2 was decreased after transfection with PGC1α-shRNA combined with CDDP treatment (**Figure 6E**). Moreover, TUNEL staining revealed that PGC1α knockdown combined with CDDP treatment significantly increased apoptosis compared with either treatment alone (**Figure 6F**), which was consistent with the *in vitro* experiments. These results further confirmed that knockdown of PGC1α combined with CDDP treatment enhanced the inhibition of NSCLC cells.

DISCUSSION

Mitochondria are the primary energy source for cellular function. Mitochondrial biosynthesis is a major cellular process that maintains mitochondrial functions (29). Numerous studies have identified the important roles of enhanced mitochondrial biosynthesis and energy metabolism in tumorigenesis and drug resistance (15). PGC1α, a major regulator of mitochondrial biogenesis, seems to perfectly reflect cellular energy requirements and the control of mitochondrial protein production, as increased demand for energy induces its expression (30). Previous studies have verified that p53 maintains mitochondrial biosynthesis by regulating mitochondrial DNA (31), therefore, p53 and PGC1α may play a common role in regulating mitochondrial biogenesis. Both Sahin et al. and Sen et al. have found that p53 negatively regulates PGC1α levels (22, 32), which was also verified in our study. We found a negative correlation between PGC1α and p53 expression in NSCLC tissues. Moreover, patients with high PGC1α expression have a short survival period. Further verification was performed by using NSCLC cells with different p53 backgrounds. The results showed that p53-deficient H1299 cells had higher expression of PGC1α and were less sensitive to CDDP. When p53 was overexpressed, the protein expression level of PGC1α and its downstream targets Tfam and Nrf1 was significantly decreased despite their increased gene expression. CHX experiments also confirmed that p53 promotes the protein degradation of PGC1α. Additionally, in our study, the proteasome inhibitors, MG132 and Epox, prevented most of the p53-mediated decrease in PGC1α protein levels. Therefore, we concluded that p53 negatively regulates PGC1α protein expression in NSCLC mostly by promoting its degradation.

Proteins are usually phosphorylated before being recognized by ubiquitin, which is easily recognized by the ubiquitin proteasome system (33). In our experiments, PGC1α phosphorylation and ubiquitination were significantly increased after p53 overexpression. Besing et al. have reported that phosphorylation by GSK-3β primes BMAL1 for ubiquitination and subsequent degradation (34). Hong et al. have found a new mechanism for the DNA damage-induced depletion of SOX9 that involves SOX9 phosphorylation by GSK-3β, thus targeting SOX9 for ubiquitination and proteasomal degradation (35). Notably, AKT inhibits GSK-3β activity by phosphorylating Ser9, while p53 inhibits AKT via PHLDA3 (18, 36, 37). In our experiments, p53 inhibited GSK-3β activity by inhibiting AKT. When combined with a GSK-3β inhibitor,

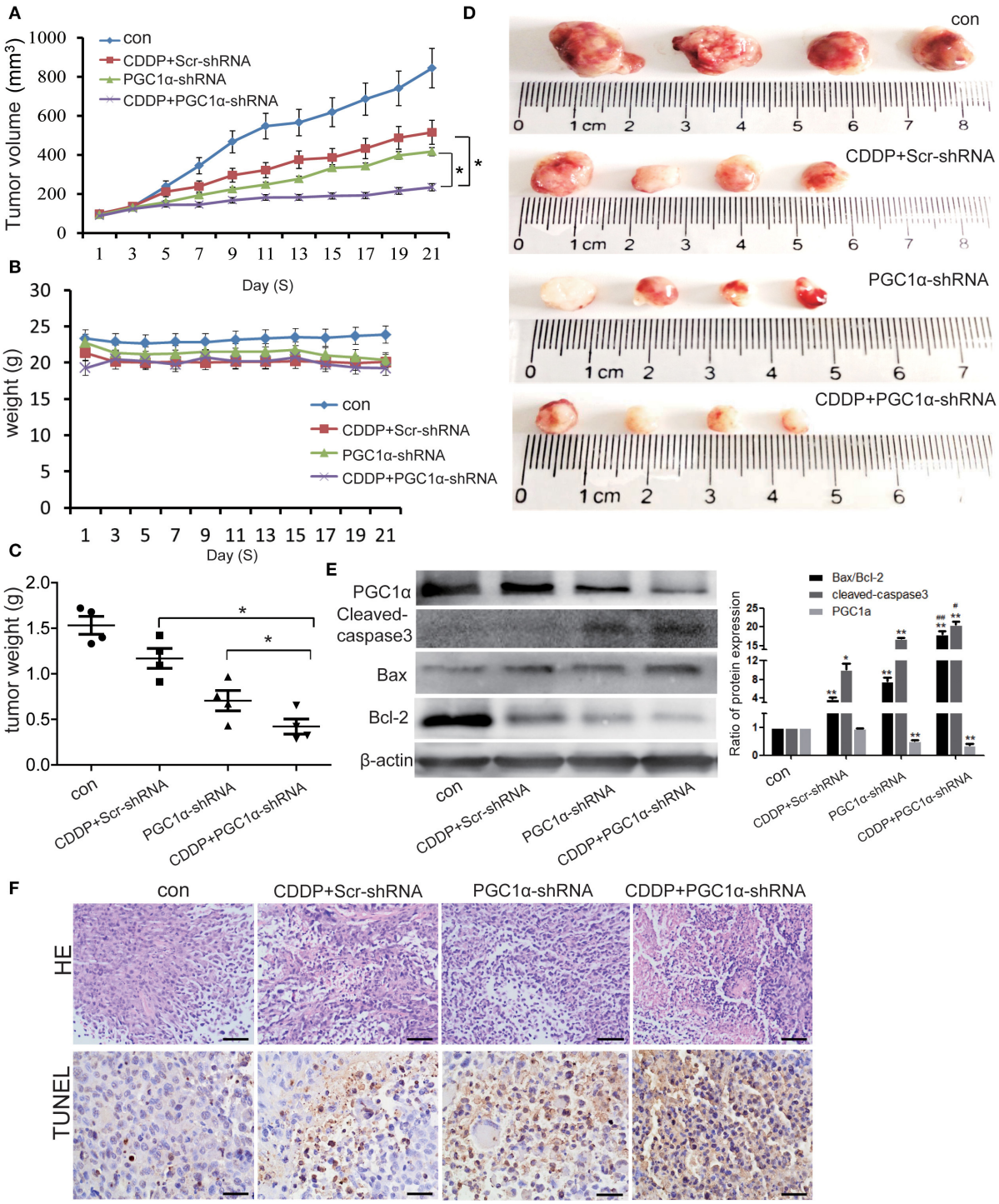

FIGURE 6 | Effects of PGC1α knockdown combined with CDDP treatment *in vivo* tumor xenografts. H1299 cells were subcutaneously implanted into nude mice. Mice were treated with 3 mg/kg CDDP and intravenously injected with attenuated Salmonella Typhi strain Ty21a harboring the PGC1α-shRNA or Scr-shRNA plasmid for 21 days ($n = 4$ per group). **(A–C)** Tumor volume and body weight were measured every 2 days. Tumor volume was determined by measuring the length and width with calipers. The wet weight of the tumors was determined at autopsy. **(D)** Images of excised tumors from each treatment group. Data are the mean ± SD, $n = 3$, $^{*}P < 0.05$, compared with the CDDP group. **(E)** Tumor tissues from the mouse xenograft model were lysed with RIPA buffer and the expression of PGC1α, Bcl2, Bax, and cleaved caspase-3 was analyzed by western blotting. Data are the mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the CDDP-shRNA or PGC1a-shRNA groups. **(F)** Representative images of the TUNEL assay performed on mouse xenograft tumor specimens. Scale bar, 50 μm.

the p53-induced increase in PGC1α phosphorylation and ubiquitination was reversed, and the decrease in PGC1α expression was reversed. Taken together, we concluded that p53 promotes the degradation of PGC1α through the AKT/GSK-3β pathway.

We demonstrated that high levels of PGC1α are associated with poor prognosis for NSCLC patients and with poor CDDP sensitivity of H1299 (p53-null) lung cancer cells. These findings are similar to the research of Vellinga et al. (14), who found that upregulating the PGC1α signaling pathway reduced the sensitivity to CDDP by transforming tumor metabolism from glycolysis to OXPHOS in colon cancer. When we expressed shRNA directed against *PGC1α* in H1299 cells, the cell sensitivity to CDDP increased, suggesting that chemoresistance of lung cancer cells with low expression of p53 is associated with high levels of PGC1α. PGC1α regulates energy metabolism and mitochondrial biogenesis primarily by coordinating with other transcription factors such as NRF1, NRF 2, and TFAM (38). In our experiments, PGC1α knockdown reduced the ATP content and the MMP in H1299 cells. This is consistent with the study of Alonso-Molero et al. on colorectal cancer, which demonstrated that decreased levels of PGC1α reduced the MMP, thus reducing chemotherapy resistance (39). A study by Do et al. using breast cancer MCF-7 cells found that decreased PGC1α expression rendered cells susceptible to oxidative stress damage by suppressing NRF2 (40). This was also demonstrated in H1299 cells, as knockdown of PGC1α increased ROS production and significantly induced apoptosis. When PGC1α knockdown was combined with CDDP treatment, these effects were stronger. When we knocked down PGC1α *in vivo* by established tumor xenografts in mice the results were consistent with the *in vitro* findings. The above experiments indicate that inhibition of PGC1α increases CDDP sensitivity and apoptosis sensitivity by reducing mitochondrial biogenesis and energy metabolism in lung cancer cells with dysfunctional p53.

In conclusion, we found that NSCLC patients with low p53 expression and high PGC1α expression had low survival rates. p53 regulates the chemotherapeutic sensitivity of tumors by regulating the stability of PGC1α via AKT/GSK-3β-mediated phosphorylation. This may be a promising therapeutic avenue for overcoming drug resistance of NSCLC patients with different p53 backgrounds.

DATA AVAILABILITY STATEMENT

The datasets in the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Clerk of Shanghai Outdo Biological Technology Co., Ltd.

AUTHOR CONTRIBUTIONS

XD performed cell research. YC and SG performed data curation. BY performed animal experiments. YLi and YLiu designed the research and supervised this study. XD wrote the manuscript. LS and JS reviewed and edited the draft. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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STRAP as a New Therapeutic Target for Poor Prognosis of Pancreatic Ductal Adenocarcinoma Patients Mainly Caused by *TP53* Mutation

*Shanshan Hu†, Xiao Chen†, Xiangxiang Xu, Chenlei Zheng, Wenqian Huang, Yi Zhou, Percy David Papa Akuetteh, Hongbao Yang, Keqing Shi, Bicheng Chen and Qiyu Zhang**

Key Laboratory of Diagnosis and Treatment of Severe Hepato-Pancreatic Diseases of Zhejiang Province, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China

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Mara Cirone, Sapienza University of Rome, Italy

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***Correspondence:**
Qiyu Zhang
qiyuz@126.com

† These authors have contributed equally to this work

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Pancreatic ductal adenocarcinoma (PDAC) has a high mortality rate and poor prognosis. *KRAS*, *TP53*, *CDKN2A*, and *SMAD4* are driver genes of PDAC and 30–75% patients have mutations in at least two of these four genes. Herein, we analyzed the relationship between these genes and prognosis of 762 patients in the absence of coexisting mutations, using data from three independent public datasets. Interestingly, we found that compared with mutations in other driver genes, *TP53* mutation plays a significant role in leading to poor prognosis of PDAC. Additionally, we found that snoRNA-mediated rRNA maturation was responsible for the progression of cancer in PDAC patients with *TP53* mutations. Inhibition of STRAP, which regulates the localization of SMN complexes and further affects the assembly of snoRNP, can effectively reduce maturation of rRNA and significantly suppress progression of *TP53*-mutant or low p53 expression pancreatic cancer cells *in vitro* and *in vivo*. Our study highlighted the actual contribution rate of driver genes to patient prognosis, enriching traditional understanding of the relationship between these genes and PDAC. We also provided a possible mechanism and a new target to combat progression of *TP53*-mutant PDAC patients.

Keywords: pancreatic ductal adenocarcinoma, *TP53*, prognosis, snoRNA, STRAP

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest human malignancies (1), with >95% mortality rate and a 5-year survival rate of less than 9% (2). It is known as the "king of cancer" due to its high degree of malignancy and currently the fourth leading cause of cancer-related deaths in the United States (2), and is expected to become the second within the next decade (3). Surgical resection is the only curative treatment for PDAC; however, this tumor is difficult to detect and quickly spreads locally or metastasizes to distant organs by the time of initial diagnosis. Therefore, less than 20% of patients have a chance of resection (4). Furthermore, most patients who undergo pancreatic resection experience local or systemic recurrences, with a median post-resection survival rate of less than 20 months (5, 6). Therefore, finding the underlying mechanisms that influence

the prognosis of PDAC is an urgent need requiring the exploration of novel adjuvant therapeutic strategies to improve the survival rate of patients.

Studies have shown that the occurrence of PDAC is caused by genetic mutations (7, 8). In recent years, with the development of next-generation sequencing technology, alterations in hundreds of genes related to axon guidance, DNA damage repair, chromatin remodelers, cell cycle regulation, and focal amplifications in druggable genes have been identified by whole genome, whole exome, and targeted deep sequencing in a large number of PDAC patients (9–11). *KRAS*, *TP53*, *CDKN2A*, and *SMAD4*, referred to as "driver genes," are the most frequently mutated genes and are well recognized as a contributing factor to pancreatic carcinogenesis (12, 13). Mutations in *KRAS* are present in more than 90% of patients (14) and are known to be related to the initiation of PDAC (15). Inactivating mutations of *TP53*, *CDKN2A*, and *SMAD4* occurred in 15–80% of PDAC patients and 30–75% had mutations in at least two of the four genes (16–18). Several studies (19–21) explored the relationship between driver genes and prognosis and largely found that these genes were associated with disease prognosis. Further, other studies (22, 23) showed that the higher the number of mutations occurring in these driver genes, the worse the prognosis, especially in patient with mutations in more than three genes. However, these studies did not take into account the possible effects of coexistence of mutations in the driver genes.

To explore the actual contribution rate of the four-driver genes to this disease, we analyzed the influence of mutation in a single gene on the prognosis of patients based on extensive sample sequencing data derived from public databases. This study also explored the possible mechanism affecting prognosis of PDAC and then investigated potential novel adjuvant therapeutic targets *in vitro* and *in vivo*.

MATERIALS AND METHODS

Patient Material Acquisition and Extraction

Data for clinical parameters, somatic mutations, and gene expression of PDAC patients were downloaded from The Cancer Genome Atlas (TCGA) Portal[1] and two other independent studies whose data were stored in the International Cancer Genome Consortium (ICGC) data portal[2].

Mutation Annotation and Filtering

All mutations obtained from public datasets were subjected to re-annotation by ANNOVAR (24) as described in our previous studies (25, 26), including cytoband, gene region, functional effect, and amino-acid change. Then, we screened mutations in the exon region because these mutations might affect the function of the protein.

[1]http://cancergenome.nih.gov/

[2]https://dcc.icgc.org/

Survival Analysis

Multivariate Cox hazard regression was used to assess the impact of some prognostic factors. Then, we used the anova() function to estimate the significance of each variable. Median survival time and cumulative survival curves were determined by the Kaplan–Meier method and differences between/among the groups were analyzed using the log-rank test. $P \leq 0.05$ was considered statistically significant.

Differential Expression Analysis of Genes

Only genes with a normalized expression value more than 0 in over 20% of the samples were considered to be expressed. Differentially expressed genes (DEGs) of different prognosis subtypes were determined with Student's two-tailed *t*-test. Since genes with expression levels that were too low reduced statistical credibility, we first excluded genes with expression levels below 5 in both of the groups used for comparison. Genes with a $P \leq 0.05$ and $|\log2FoldChange| \geq 1$ were defined as differential genes. Simultaneously, RankCompV2 (27), a rank-based algorithm, was used for differential expression analysis and utilized to calculate DEGs with default parameters. This method was not affected by the level of gene expression.

Functional Enrichment Analysis

To identify enriched pathways and gene ontologies of gene sets, we performed enrichment analysis using the R package ClusterProfiler. For the pathway analysis, we used the pathway annotations package ReactomePA provided by Reactome Pathway Database. GO gene set collections were obtained from GO.db package. We performed Fisher's exact test and permutation test to calculate *P* and OR values for enrichment analysis of the family genes or cluster genes. The permutation test was based on random sampling, as in our previous study (26). Specifically, we calculated the *P* by comparing the number of differential genes in this family/cluster to the number of genes from the family/cluster of 1,000,000 simulated datasets. Each simulated dataset included the same number of total DEGs by random sampling.

Cell Lines

Mutant background of the pancreatic cancer cell lines was queried by Cancer Cell Line Encyclopedia (CCLE)[3].

PANC-1, Patu-8988, and PANC-0327 cells were purchased from the American Type Culture Collection (Manassas, VA, United States). KP4 cell line was obtained from the Riken BioResource Center Cell Bank (Ibaraki, Japan). All the cell lines were cultured in either DMEM or RPMI-1640 media supplemented with 10% fetal bovine serum, and were free of mycoplasmas and authenticated by polymorphic short tandem repeat loci before use.

Cell lines stably overexpressing human p53 in *TP53*-mutant cells or p53-knockdown in *TP53* non-mutant cells were generated by infecting cells with lentiviruses expressing p53 or p53 shRNA (MOI = 10; GeneChem Co. Ltd., Shanghai,

[3]https://portals.broadinstitute.org/ccle

China), respectively. STRAP-knockdown cells were generated by infecting cells with lentiviruses expressing two specific STRAP shRNAs (MOI = 10; GeneChem Co. Ltd.). Cells infected with lentiviruses expressing control empty vector or shRNA were used as controls. We selected successfully infected cells with puromycin (1 μg/ml) for 7 days.

Western Blot Analysis

Western blot analysis was performed as described previously (28, 29). The following commercially available antibodies were used in this study: GAPDH (Cell Signaling Technology, Shanghai, China; catalog no. 2118), p53 (ProteinTech, Wuhan, China; catalog no. 10442-1-AP) and STRAP (ProteinTech; catalog no. 18277-1-AP).

qPCR

Total RNA was isolated using TRIzol reagent (Life Technologies, Shanghai, China) and reverse-transcribed using the M-MLV reverse transcription kit (Promega, Madison, WI, United States). qPCR was carried out in an ABI 7500 Fast instrument (Life Technologies) using the SYBR Premix Ex Taq kit (TaKaRa, Dalian, China).

Ribosomal RNA Processing Analyses

We performed qPCR to evaluate rRNA processing. Gene-specific primers of 18S and 28S rRNA (**Supplementary Table 1**) and the calculation method for the fraction of unprocessed rRNA were determined as described previously by Cao (30). Specifically, the unprocessed rate of 18S rRNA was the averages of primer pairs 4/3 (unprocessed) over 2/1 (total) and primer pairs 6/5 (unprocessed) over 2/1 (total), and that for 28S rRNA was the averages of primer pairs d/c (unprocessed) over b/a (total) and primer pairs f/e (unprocessed) over b/a (total).

Cell Proliferation, Migration, and Invasion Assays

Lentivirus-transfected pancreatic cancer cells were plated into 96-well plates at a density of 3×10^3 cells per well to test cell proliferation. The Cell Counting Kit-8 (CCK-8) reagent (Dojindo, Kyushu Island, Japan) was used to detect cell viability every 24 h for 3 days. The OD value (450 nm) was recorded to generate a cell proliferation curve.

Wound-healing assays were used to assess the migration ability of cells. Transfected cells were seeded into 12-well plates and then cultured for 24 h until 95% confluence. The confluent monolayer in each well was created using a 1,000 μl pipette tip and cultured for 48 h. Cells were photographed at 0, 24, and 48 h under a Nikon Eclipse TE2000-U Inverted Microscope (Nikon, Tokyo, Japan).

For the invasion assay, 2×10^4 cells per well were plated into the upper chamber of a 24-well Transwell chamber (Corning, NY, United States) and coated with Matrigel and serum-free medium. Then, 500 μl complete medium with 10% FBS was added into the lower chamber. Cell migration through the Matrigel substrate was assessed after 24 h by fixing it in 4% paraformaldehyde, staining with 1% crystal violet (Sigma), and counting the migrated cells by selecting five fields at random under a light microscope.

Animal Studies

All animal studies and procedures were approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University. Tumor xenografts were generated by adding 5×10^6 Patu-8988 cells with p53 overexpression (LV-pP53) or control empty vector (LV-Con) and 5×10^6 KP4 cells with p53-knockdown (LV-shP53) or control shRNA (LV-shCon) to 100 μl PBS, and then subcutaneously injected into each flank of 6-week-old female athymic BALB/c nude mice. When the volume of the tumors was about 100 mm^3, mice were randomly assigned to two groups (5 mice/per group) and then received an intratumoral injection of shSTRAP-1 or shCon at a titer of 10^7 TU in 10 μl PBS every 3 days, which was repeated three times. The volume of tumors was calculated with the following formula: $V = (Width^2 \times Length)/2$. Mice were sacrificed 35 days following tumor injection. The investigator was not blinded to group allocation during the experiment but was blinded when assessing the xenograft tumor volumes following euthanasia of the mice.

RESULTS

Data Collection

In total, we retrieved detailed clinical information from 923 PDAC patients, which included 784 somatic mutations, and 279 RNA sequences from three independent PDAC-related studies, including TCGA, and two other independent studies stored in ICGC (PACA-AU, PACA-CA) (**Supplementary Table 2**). There were 762 PDAC samples with both survival information and somatic mutation data. Patients of TCGA, PACA-AU, and PACA-CA were from the United States, Australia, and Canada, respectively. Data utilized from all three countries included 154, 461, and 308 follow-up survival data, 133, 391, and 260 somatic mutation information, and 142, 91, and 46 RNA sequence data, respectively.

Multivariate Analysis of the Clinical Parameters Regarding the Prognosis of Patients With PDAC

Due to the lack of detailed clinical data, we only assessed the impact of some parameters on prognosis (**Supplementary Table 3**). Using multivariate Cox analysis, we found no difference in survival rates among patients in the three databases ($P = 0.58$). Further, we analyzed the effects of gender and age on the prognosis of patients and found no difference. However, the number of mutations in driver genes had a significant effect on the prognosis of patients ($P = 0.0028$), which is consistent with previous reports (19–21).

Next, we analyzed the mutation frequency of the driver genes in the patients and found that it was consistent with previous results: more than 90% (90.43%) of patients had *KRAS* mutations. Patients carrying *TP53*, *SMAD4*, and *CDKN2A* mutations were

69.13, 23.21, and 20.66%, respectively. Moreover, nearly 75% (74.74%) of patients were carrying more than two mutations at the same time. Among them, 98.63% of patients had *KRAS* mutations (**Supplementary Table 4**).

Analysis of Prognosis in Patients With Mutations in Driver Genes

Since the *KRAS* mutation is present in almost all patients and is the initiator of the disease, we analyzed the prognosis of patients with only *KRAS* mutations and those without any driver gene mutations and found no difference between the two groups (**Figure 1A**). Therefore, when considering the contribution of mutations in the other three driver genes to prognosis, activation of *KRAS* was used as the basis; hence we used it as the control group.

When analyzing the effects of *TP53*, *SMAD4*, and *CDKN2A* mutations on the prognosis of patients, we first analyzed the overall survival of patients with mutations in only one of the three driver genes based on the activation of *KRAS* and found that only patients with *TP53* mutations were significantly different from the control group (**Figure 1B**). Under conditions of coexistence of mutations in two or three driver genes, we also found that only patients with *TP53* mutations simultaneously had a significant difference in prognosis compared to the control group (**Figure 1C**). This suggests that *TP53* may play a significant role in affecting patient prognosis.

To further confirm whether the influence of other driver gene mutations on prognosis was due to the coexistence of *TP53* mutations, we re-analyzed the relationship between *CDKN2A* and *SMAD4* mutations in relation to prognosis. Consistent with the original conclusion, we found that the prognosis of patients with *CDKN2A* or *SMAD4* mutations was significantly worse than that of patients without mutations when the *TP53* mutation status was not considered (**Figures 1D,E**). However, when patients with *TP53* mutations were excluded, the prognosis between the two groups of patients exhibited no significant difference (**Figures 1D,E**).

In summary, the above results indicated that *TP53* is the real key factor leading to poor prognosis. The prognostic analysis revealed that the prognosis of patients with *TP53* mutations was significantly reduced compared to that of patients without *TP53* mutation after *KRAS* activation (**Figure 1F**).

TP53 Affects the Progress of Pancreatic Cancer Cell Lines *in vitro* and *in vivo*

We selected pancreatic cancer cell lines PANC1 and Patu-8988 with both *KRAS* and *TP53* mutations and KP-4 and PANC-0327 with only *KRAS* mutations to verify the dependence of pancreatic cancer survival on *TP53* (**Supplementary Table 5**). CCK8 proliferation assay results demonstrated that overexpression of *TP53* (LV-pP53) in PANC1 and Patu-8988 displayed a significant decrease in cell proliferation compared with that in the control group (LV-Con) (**Figures 2A,B**). Wound-healing assays indicated that the migration distance of the LV-pP53 group was shorter than that of the control group (**Figure 2C**). In parallel, the results of the Transwell invasion assay showed that the invasion ability of LV-pP53 was lower than that of the control group (**Figure 2D**). Similarly, we also found that overexpressing p53 could effectively suppress xenograft tumor growth (**Figure 2E**). However, compared with those in the control group (LV-shCon), the proliferation, migration, invasion, and xenograft tumor growth of p53-knockdown in KP-4 and PANC-0327 (LV-shP53) were promoted (**Figure 3**).

Analysis of Differentially Expressed Genes and Their Functional Pathways

In order to find out the mechanism by which *TP53* mutation affects prognosis, we divided patients from public sources into two groups based on *TP53* mutation status: *TP53*_mut and *TP53*_wt. Then, we analyzed DEGs by Student t-test and RankCompV2. A total of 90 DEGs were identified by Student's t-test, including 73 upregulated and 17 downregulated. RankCompV2 also found 90 DEGs, with 60 upregulated and 30 downregulated (**Supplementary Table 6**). We performed GO and pathway enrichment analyses to further investigate functional pathways associated with the DEGs. Results showed that genes were enriched in several biological processes and pathways that are known to be associated with nucleosome assembly and the transcriptional regulation of genes, such as chromatin assembly (GO: 0031497), DNA packaging (GO: 0006323), chromatin silencing (GO: 0006342) and RNA Polymerase I Promoter Opening (R-HSA-73728), HDACs deacetylate histones (R-HSA-3214815), and DNA methylation (R-HSA-5334118) (**Supplementary Figure 1** and **Supplementary Tables 7, 8**). More importantly, we noted that the functional pathways were involved in the regulation of rDNA (chromatin silencing at rDNA; GO: 0000183) and rRNA expression (SIRT1 negatively regulated rRNA expression; R-HSA-427359, NoRC negatively regulated rRNA expression; R-HSA-427413, B-WICH complex positively regulated rRNA expression; R-HSA-5250924) as well as the high enrichment of Cajal bodies RNAs and the snoRNA family genes (**Table 1**).

TP53 Affects the Maturation of Ribosomal RNAs

In humans, snoRNAs are primarily responsible for the modification and maturation of ribosomal RNAs (rRNAs) (31). Global control of protein synthesis is crucial for cancer development and progression, as highly proliferating cancer cells require increased protein synthesis (32); therefore, more rRNAs may be needed to participate in protein synthesis. Thus, we hypothesized that snoRNA-mediated rRNA maturation might be a cause of cancer progression in patients with *TP53* mutations. The prognostic analysis showed that upregulated snoRNA gene expression was significantly associated with poor prognosis (**Figure 4A**). qPCR analysis showed that the proportion of mature 18S and 28S rRNA was significantly decreased in the p53 overexpressing PANC1 and Patu-8988 *in vitro* and *in vivo* (**Figures 4B,D**), whereas p53 knockdown in KP-4 and PANC-0327 promoted the maturation of 18S and 28S rRNA *in vitro* and *in vivo* (**Figures 4C,E**).

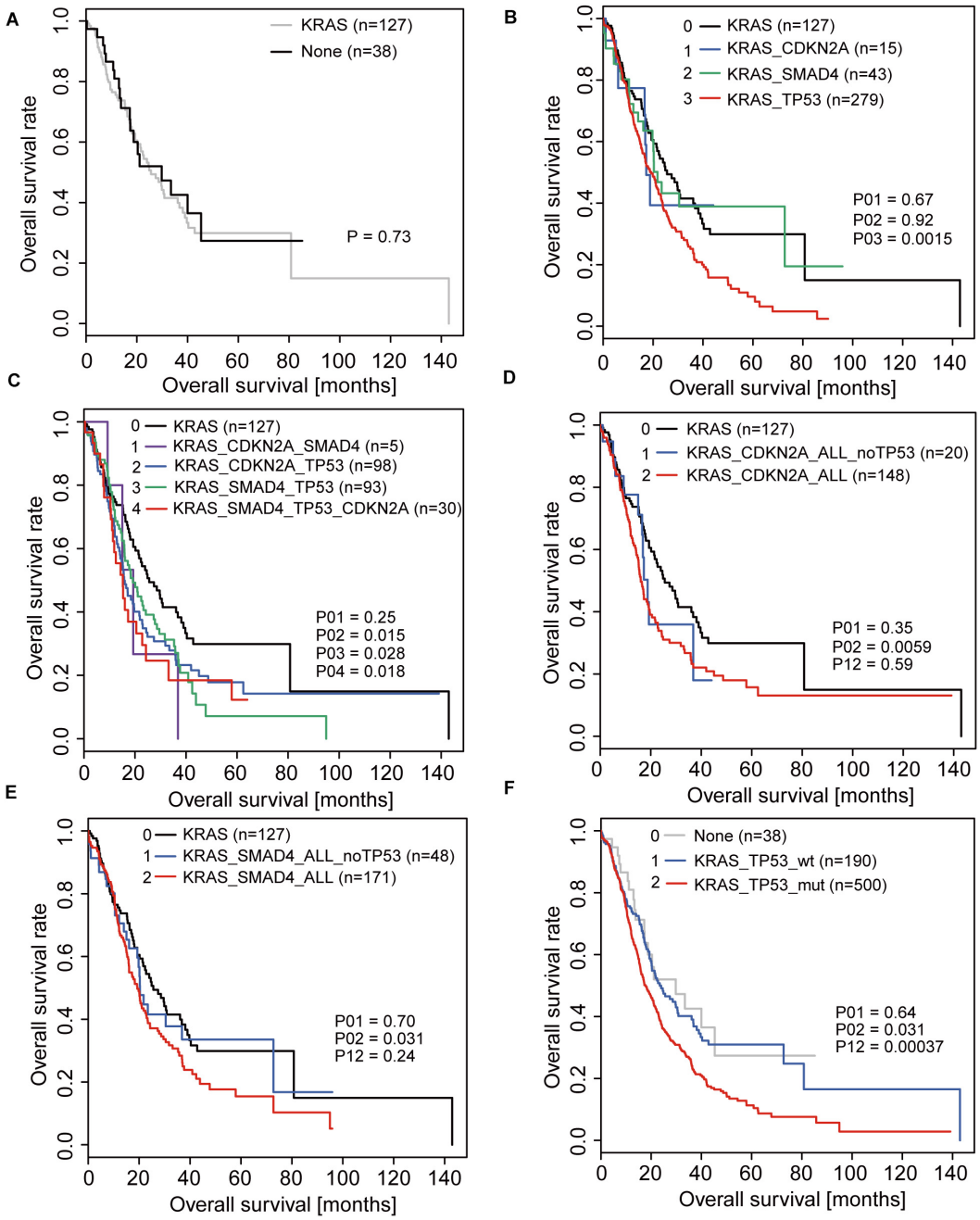


FIGURE 1 | Overall survival (OS) analyses in PDAC patients correlated with driver gene mutations. **(A)** OS of patients with *KRAS* mutations only and without any driver gene mutations. **(B)** OS of patients with mutations in only one driver gene based on the activation of *KRAS*. **(C)** OS of patients with coexistence of mutations in driver genes based on the activation of *KRAS*. **(D,E)** OS of patients with *CDKN2A* mutations **(D)** or *SMAD4* mutations **(E)** when considering and not considering *TP53* mutations. **(F)** OS of patients with and without *TP53* mutations.

Knockdown of STRAP Effectively Blocks the Progression of Pancreatic Cancer Cells With Low p53 Expression *in vitro* and *in vivo*

STRAP, also known as UNRIP, is a serine/threonine kinase receptor-associated protein. Krastev et al. (33) found that STRAP affected the localization of SMN complex in a p53-independent manner, which in turn affected the assembly of snoRNP. Inhibition of STRAP could effectively reduce the proliferation and migration of *TP53*-mutant colon cancer cells without affecting the growth of *TP53* non-mutated cancer cells (33). Our prognostic analysis of public data showed that downregulated STRAP significantly improved the prognosis of

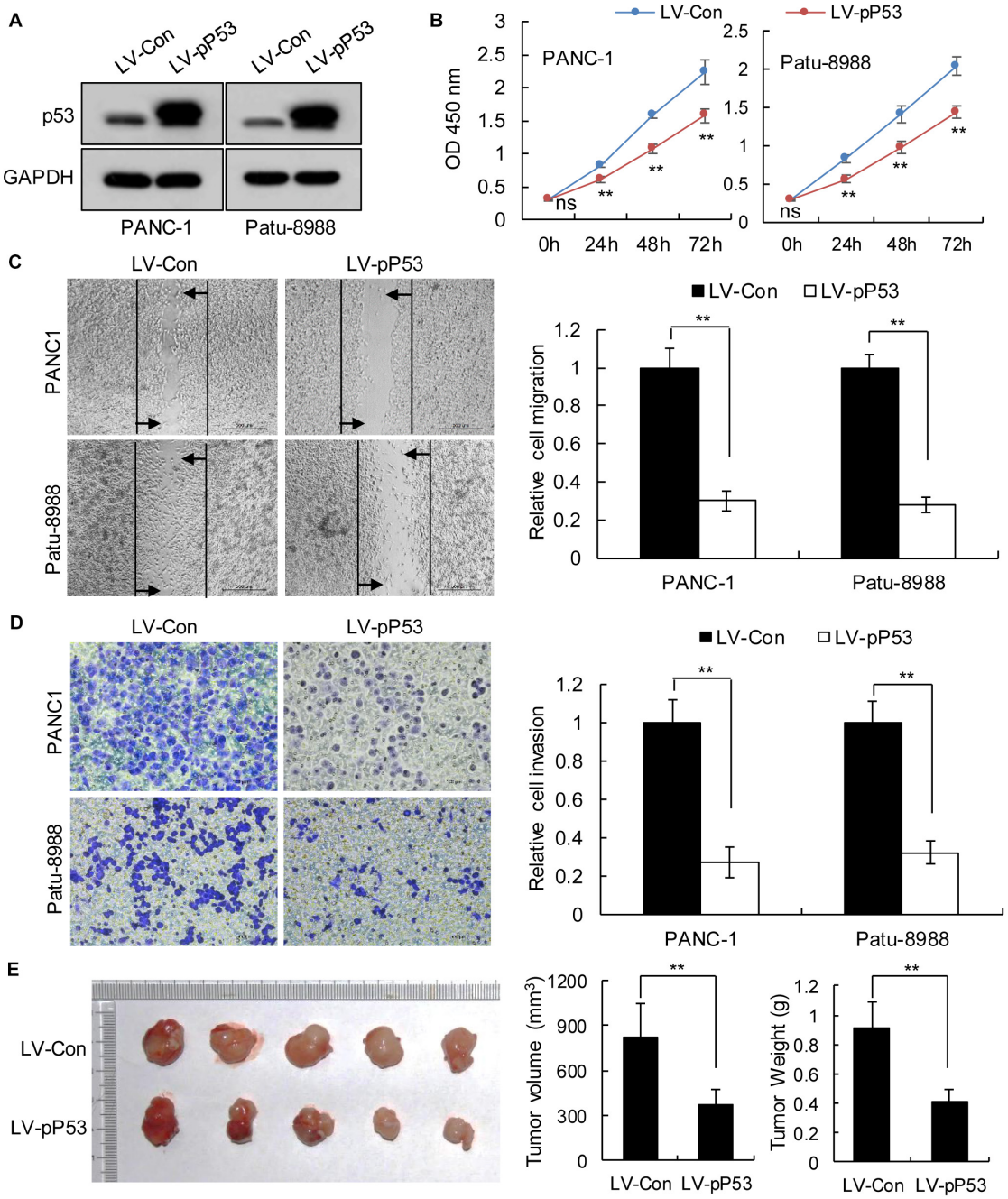


FIGURE 2 | Overexpression of p53 in *TP53*-mutant pancreatic cancer cells suppressed cancer progression *in vitro* and *in vivo*. PANC-1 and Patu-8988 cells were infected with control or over-expressing *TP53* lentiviruses. **(A)** p53 expression was analyzed by western blotting. **(B)** The cell proliferation assay was performed at the indicated time points. **(C)** Representative micrographs of cell migration assays at 48 h (left) and quantification results (right). **(D)** Representative micrographs of cell invasion assays (left) and quantification results (right). Data in panels **(B–D)** are shown as the mean ± SEM of 3 independent experiments. **(E)** Representative images, volumes and weights of subcutaneous xenografts of Patu-8988 cells with overexpressing p53 or control. Data represent means ± SEM for 5 mice per group. $^{**}P < 0.01$.

patients with PDAC (**Supplementary Figure 2A**), with the effect being better in the *TP53* mutant state than in the non-mutated state (**Supplementary Figures 2B,C**).

To verify whether inhibition of STRAP was effective against *TP53*-mutant pancreatic cancer cells by inhibiting snoRNA-mediated rRNA maturation, we successfully constructed STRAP-interfering stable cell lines based on p53 overexpression (**Figure 5A** and **Supplementary Figure 3A**) or p53-knockdown (**Figure 6A** and **Supplementary Figure 4A**). Both *in vitro and in vivo*, knockdown of STRAP in the *TP53*

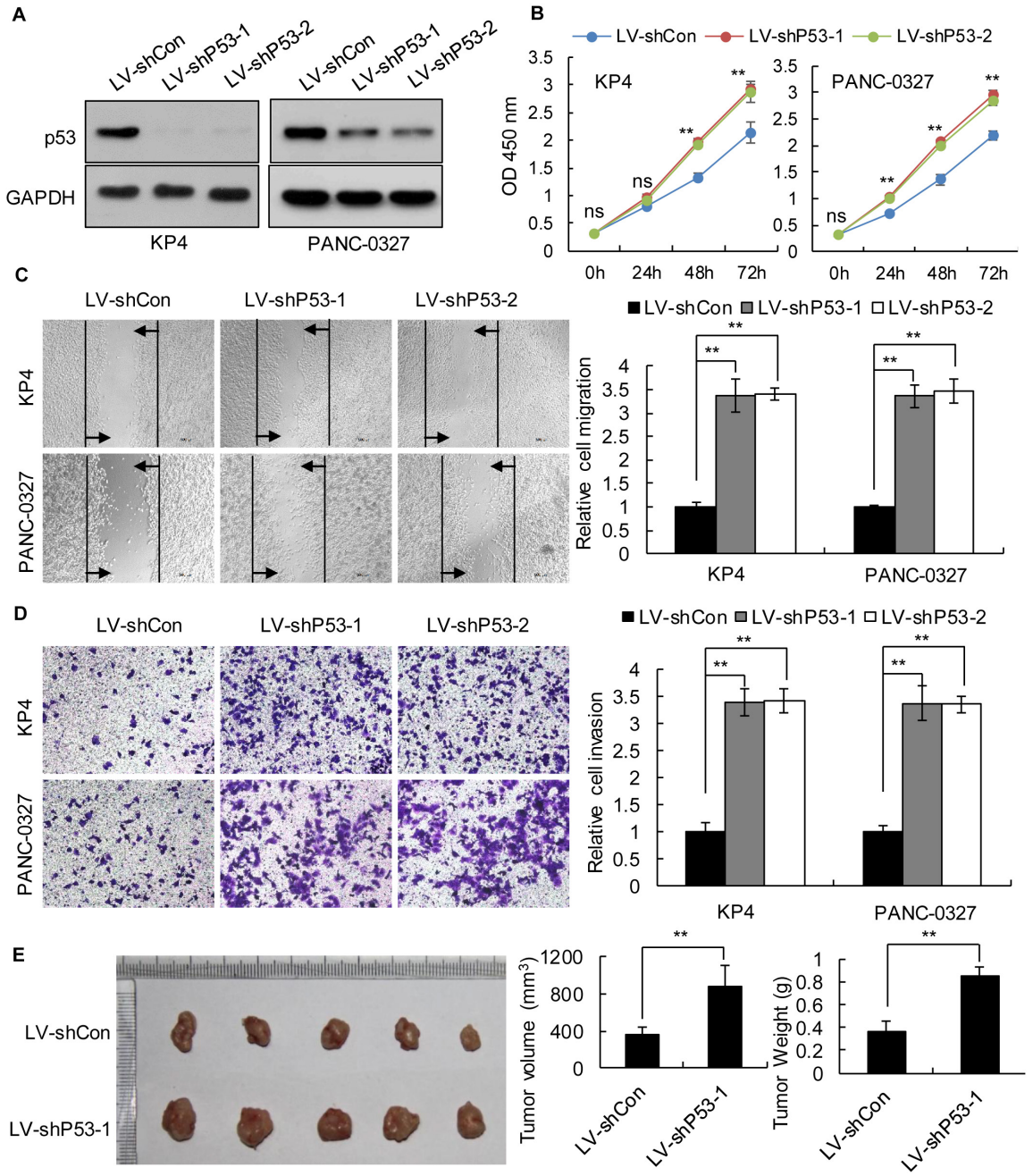


FIGURE 3 | Knockdown of p53 in *TP53* non-mutant pancreatic cancer cells promoted cancer progression *in vitro* and *in vivo*. KP4 and PANC-0327 cells were infected with control or p53-knockdown lentiviruses. **(A)** p53 expression was analyzed by western blotting. **(B)** The cell proliferation assay was performed at the indicated time points. **(C)** Representative micrographs of cell migration assays at 48 h (left) and quantification results (right). **(D)** Representative micrographs of cell invasion assays (left) and quantification results (right). Data in panels **(B–D)** are shown as the mean ± SEM of 3 independent experiments. **(E)** Representative images, volumes and weights of subcutaneous xenografts of KP4 cells with p53 knockdown or control. Data represent means ± SEM for 5 mice per group. $^{**}P < 0.01$.

mutant state (**Figures 5B–G** and **Supplementary Figures 3B–E**) or p53-knockdown (**Figures 6B–G** and **Supplementary Figures 4B–E**) did indeed inhibit rRNA maturation and could effectively inhibit the development of cancer *in vitro* and *in vivo*, but there was no significant effect on the high p53 expression cell lines.

DISCUSSION

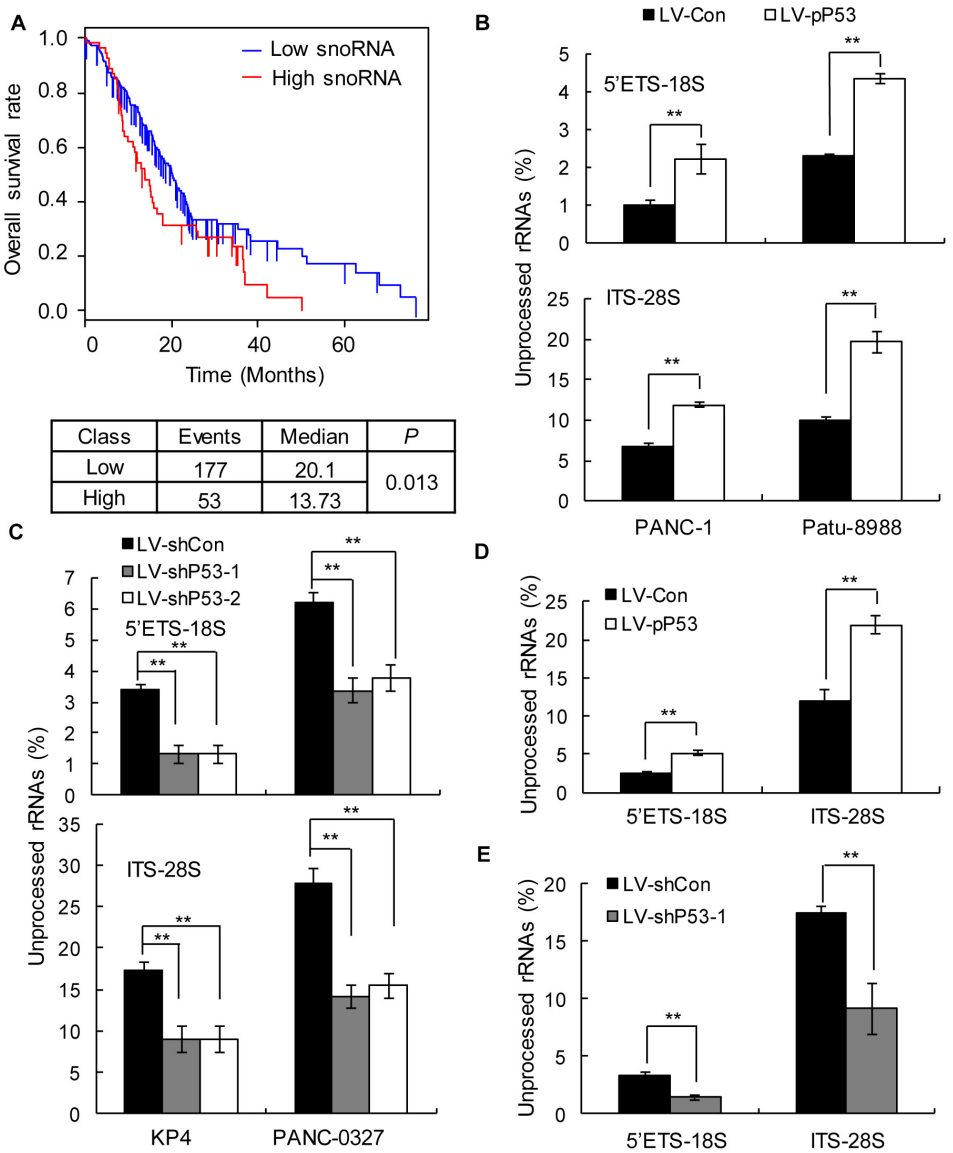
As the "king of cancer," PDAC has a high mortality rate and poor prognosis (2). Therefore, it is of great significance to search for the key factors that affect the prognosis of PDAC patients and effective adjuvant treatment measures for clinical treatment

TABLE 1 | Functional enrichment analysis of snoRNA family genes and related cluster genes.

	Background	T test	Fisher's exact test		Permutation test		Background	RankCompV2	Fisher's exact test		Permutation test	
Total	**11789**	**90**	***P***	**OR**	***P***	**OR**	**16859**	**90**	***P***	**OR**	***P***	**OR**
box H/ACA snoRNAs	28	10	1.48e-13	52.39 (21.94–115.58)	<1.00e-06	48.45 (48.25–48.66)	48	4	1.66e-04	16.28 (4.17–45.88)	1.19e-04	15.62 (15.56–15.68)
box C/D snoRNAs	5	2	1.16e-03	53.46 (5.02–330.76)	5.48e-04	52.27 (51.76–52.80)	7	0	1.00	0.00 (0.00–131.85)	1.00	0.00 (0.00–0.00)
Total snoRNA	33	12	3.93e-16	54.69 (24.75–113.52)	1.75e-03	13.66 (13.61–13.71)	55	4	2.71e-04	14.20 (3.66–39.78)	1.75e-04	13.66 (13.61–13.71)
Cajal bodies RNAs	7	2	1.97e-03	38.17 (3.82–205.03)	1.128e-03	37.62 (37.31–37.94)	13	3	7.71e-05	44.60 (8.01–166.48)	2.80e-05	43.19 (42.87–43.51)

of PDAC and increase the prognostic survival rate of patients. It is known that *KRAS*, *TP53*, *CDKN2A* and *SMAD4* play an important role in the development of PDAC (12, 13), and are significantly associated with the prognostic survival of patients (19–23). However, mutations in at least two of these four driver genes are present in 30–75% of patients with PDAC (16–18). Is there a bias in the contribution of driver genes to patient survival? Will the relationship between a driver gene and patient prognosis be affected by mutations in other driver genes? This has not been noticed in previous research.

As the most frequent mutated genes in PDAC, *KRAS*, *TP53*, *CDKN2A* and *SMAD4* have been well explored in many studies regarding their relationship with the prognosis of PDAC patients (19–23). Herein, we analyzed data from 762 patients with PDAC and explored the actual contribution rate of four driver genes to prognosis in the absence of coexisting mutations by combining multiple statistical and bioinformatics methods. This study differs from previous studies in that it considers the large number of coexisting mutations of the four driver genes in patients with PDAC, which may partially obscure the true contribution of each gene to patient prognostic survival rate. Interestingly, we found that compared with patients with other driver gene mutations, only patients with *TP53* mutations simultaneously had a significantly lower prognosis than patients in the control group. Additionally, we found that the relationship between other driver gene mutations and prognosis will be affected by the existence of *TP53* mutations. This reminds us that when studying the relationship between other driver genes and PDAC in the future, it is necessary to consider that the coexistence of *TP53* mutations may have an impact on the results. Through *in vitro* and *in vivo* experiments, we also verified the necessity of p53 for the growth of pancreatic cancer. This finding highlighted the actual contribution rate and enriched the traditional understanding of the relationship between these genes and prognosis. However, the results were only based on the univariate analysis of driver gene mutation, other clinical parameters such as age and gender might also have some influence on the result. This requires in-depth research to enrich our conclusions in the future. In this study, we also explored the possible mechanism of p53 affecting patients prognosis. We found that compared with *TP53* non-mutant patients, *TP53*-mutant patients have a high expression of snoRNA family genes, and their DEGs are significantly enriched in several biological processes and pathways related to the regulation of rDNA and rRNA. SnoRNAs are a family of conserved RNAs, concentrated in Cajal bodies or nucleoli where they either function in the modification of rRNAs or participate in the processing of rRNAs during ribosomal subunit maturation (34). Many studies have shown that snoRNA is abnormally regulated in tumors (35–47), and snoRNA or snoRNA host genes can affect the proliferation, apoptosis, invasion and migration of cancer cells (37–47). Okugawa et al. and Mei et al. found snoRA42 enhance the proliferation, migration, invasion in colorectal cancer (CRC) and Lung cancer (41, 42). Fang et al. found snoRD126 activate the PI3k-AKT pathway to facilitate hepatocellular carcinoma (HCC) and CRC cell growth (43). Cui et al. found snoRA23 promote growth and metastasis by regulates expression of SYNE2



Class	Events	Median	*P*
Low	177	20.1	0.013
High	53	13.73	

FIGURE 4 | p53 expression is associated with snoRNA-mediated ribosome maturation. **(A)** Kaplan–Meier survival curves for PDAC patients according to the snoRNA family genes in tumor tissues, and significance was calculated using the log-rank test. **(B,D)** Overexpression of p53 in *TP53*-mutant cells *in vitro* **(B)** and *in vivo* **(D)** promotes rRNA processing by detecting 5'ETS-18S and ITS–28S using quantitative reverse transcriptase PCR assays in PANC-1 (left) and Patu-8988 cells (right). **(C,E)** Knockdown p53 in *TP53* non-mutant cells *in vitro* **(C)** and *in vivo* **(E)** suppresses rRNA processing by detecting 5'ETS-18S and ITS–28S using quantitative reverse transcriptase PCR assays in KP4 (left) and PANC-0327 cells (right). The data are shown as the mean ± SEM of 3 independent experiments. $^{**}P < 0.01$.

in pancreatic ductal adenocarcinoma (PDAC) (38). Valleron et al. found snoRD112-114 affects Rb/p16 cell cycle regulation to promote cell growth in acute promyelocytic leukemia (APL) (44). Siprashvili et al. found snoRD50A and snoRD50B activate the K-Ras/B-Raf-MEK-ERK pathway to facilitate the proliferation of tumor cells (45). Wu et al. found snoRNA Sf-15 can participate in apoptosis through regulating the expression of Ca2 + -induced cell death pathway gene Cn in Sf9 cells (46). Xia et al. found SNORD44 activate the caspase-dependent apoptosis pathway to facilitate the apoptosis in glioma cells (47). However, these studies are focused on the function of a single snoRNA. In this study, we found that snoRNA family genes are dysregulated expressed in clusters, rather than the disorder of a single snoRNA gene. Therefore, we speculate that snoRNA-mediated rRNA maturation, which is the unified function of snoRNA, might be a cause of cancer progression in patients with *TP53* mutations. Our prognostic analysis showed that upregulated snoRNA was significantly associated with poor prognosis in patients with PDAC. Experiments *in vitro* and *in vivo* have shown that the proportion of mature 18S rRNA and 28S rRNA is significantly reduced in p53 overexpressed PANC-1 and Patu-8988 pancreatic cancer cell lines, and knockdown of p53 in KP4 and PANC-0327

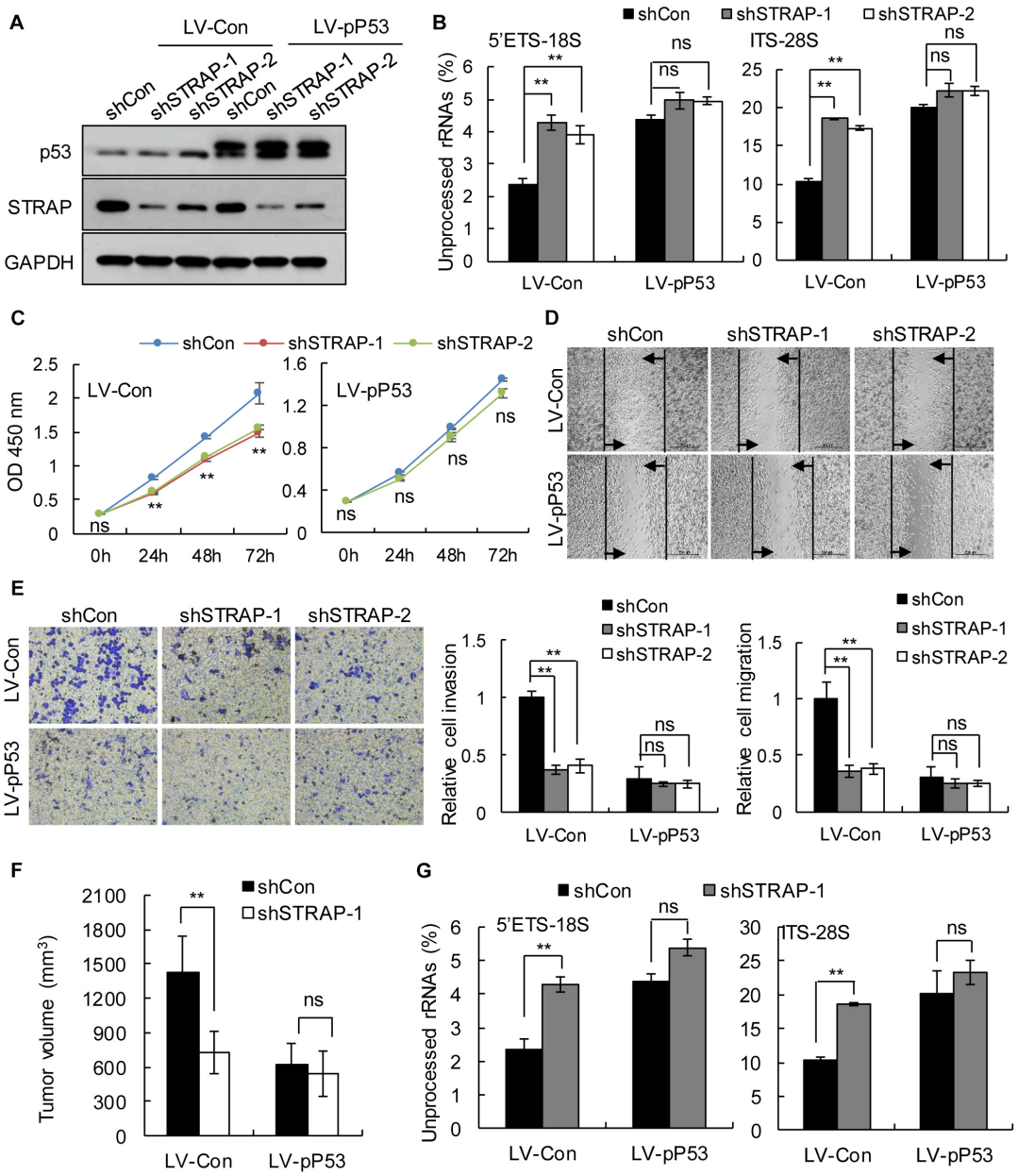


FIGURE 5 | STRAP knockdown suppressed progression of *TP53*-mutant Patu-8988 cells by inhibiting snoRNA-mediated rRNA maturation. Patu-8988 cells with p53 overexpression or control vector were infected with control or STRAP-knockdown lentiviruses. **(A)** p53 and STRAP expression were analyzed by western blotting. **(B,G)** rRNA processing *in vitro* **(B)** and *in vivo* **(G)** by detecting 5'ETS-18S and ITS–28S using quantitative reverse transcriptase PCR assays. The data are shown as the mean ± SEM of 3 independent experiments. **(C)** The cell proliferation assay was performed at the indicated time points. **(D)** Representative micrographs of cell migration assays at 48 h (top) and quantification results (bottom). **(E)** Representative micrographs of cell invasion assays (left) and quantification results (right). Data in panels **(C–E)** are shown as the mean ± SEM of 3 independent experiments. **(F)** Representative volumes of subcutaneous xenografts of Patu-8988 cells with overexpressing p53 or control injected intratumorally with control or STRAP- knockdown lentivirus. Data represent means ± SEM for 5 mice per group. $^{**}P < 0.01$.

pancreatic cancer cell lines promoted the maturation of 18S rRNA and 28S rRNA. These results indicate that snoRNA-mediated rRNA maturation may be a possible mechanism for the progression of cancer in PDAC patients with *TP53* mutations, but we believe that snoRNA-mediated rRNA maturation is not simply a surrogate for proliferation rate, other targets and pathways affecting the proliferation of *TP53* mutant pancreatic cancer cells need further exploration, which is. a direction worthy of in-depth study in the future.

STRAP, a protein containing WD40 (48), is thought to play an important role in regulating eukaryotic cell growth and development by inhibiting transforming growth factor-beta (TGF-β) and various other signaling pathways (49–51). Recent studies have shown that overexpression and misregulation of

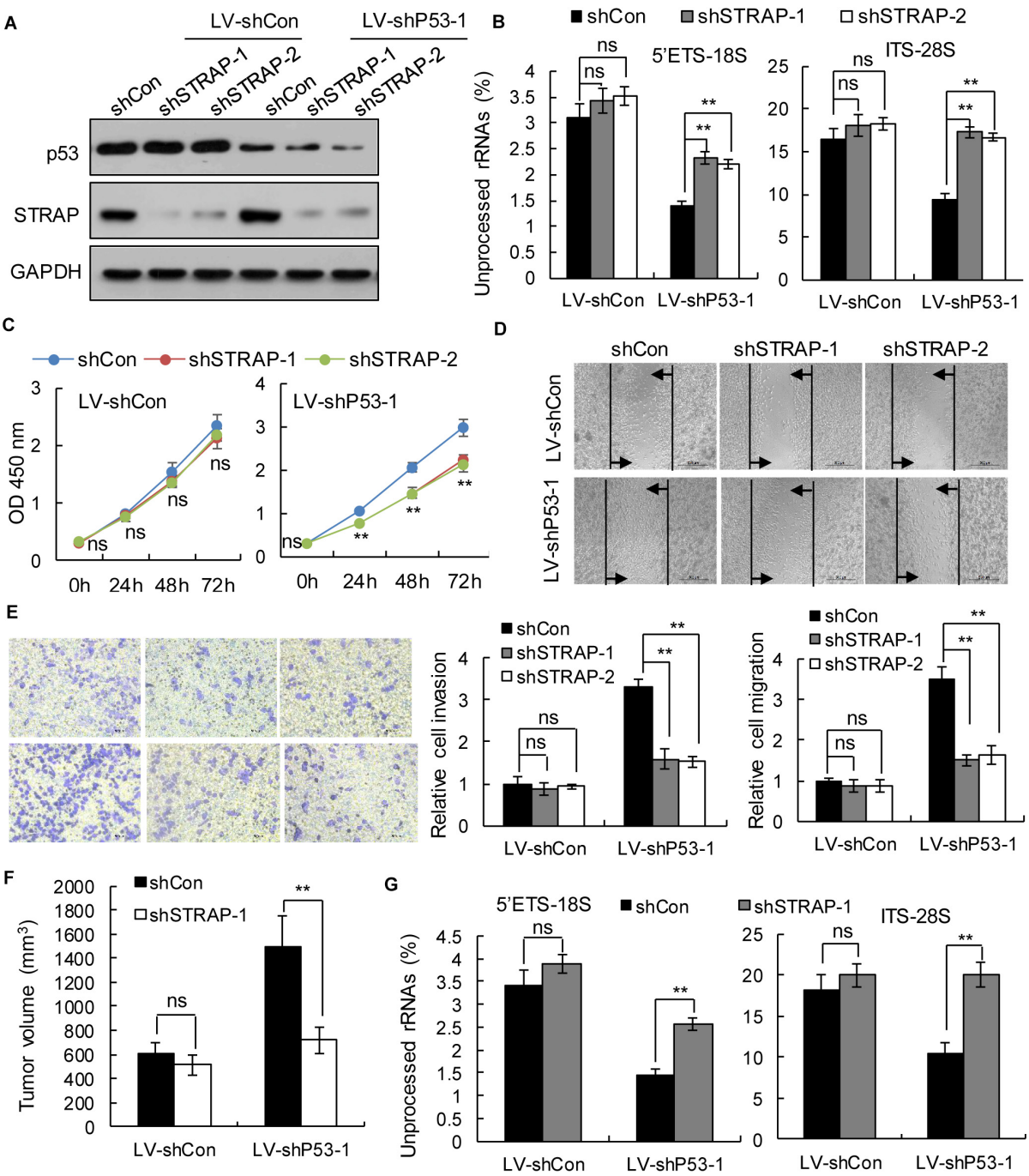


FIGURE 6 | STRAP knockdown suppressed progression of p53-knockdown KP4 cells by inhibiting snoRNA-mediated rRNA maturation. KP4 cells with p53 knockdown or control vector were infected with control or STRAP-knockdown lentiviruses. **(A)** p53 and STRAP expression were analyzed by western blotting. **(B,G)** rRNA processing *in vitro* **(B)** and *in vivo* **(G)** by detecting 5'ETS-18S and ITS–28S using quantitative reverse transcriptase PCR assays. The data are shown as the mean ± SEM of 3 independent experiments. **(C)** The cell proliferation assay was performed at the indicated time points. **(D)** Representative micrographs of cell migration assays at 48 h (top) and quantification results (bottom). **(E)** Representative micrographs of cell invasion assays (left) and quantification results (right). Data in panels **(C–E)** are shown as the mean ± SEM of 3 independent experiments. **(F)** Representative volumes of subcutaneous xenografts of KP4 cells with p53 knockdown or control injected intratumorally with control or STRAP- knockdown lentivirus. Data represent means ± SEM for 5 mice per group. $^{**}P < 0.01$.

STRAP are associated with the development of multiple cancers (52–54) and thus it could be considered a new therapeutic target for cancer. Our prognostic analysis showed that the expression of STRAP was significantly associated with the prognosis of PDAC patients. Krastev et al. (33) found that *TP53* can regulate immature snoRNPs into the Cajal body by regulating the level

of NOLC1, and then immature snoRNPs interact with COIL and SMN to assemble mature snoRNPs. STRAP plays an important role in regulating the cellular localization of SMN complex, which is necessary for the SMN complex to enter Cajal body (55, 56). As a downstream concomitant factor affecting snoRNP assembly by *TP53*, the expression of STRAP is currently known to be independent of p53 expression. Our study also found that there was no significant difference in STRAP expression between PDAC patients with *TP53* mutation and patients with no *TP53* mutation. Krastev et al. (33) also showed that knocking down STRAP had no effect on the growth of *TP53* wild-type colon cancer cells, whereas expression of STRAP was required for efficient growth of *TP53* knockout colon cancer cells. This shows that STRAP as a target for adjuvant therapy may provide a huge advantage in terms of mitigating toxic and side effects on *TP53* non-mutated normal cells. Based on this, we successfully constructed STRAP-interfering pancreatic cancer cell lines with STRAP shRNA lentivirus, and verified their effects *in vitro* and *in vivo*. We found that knocking down STRAP could effectively inhibit rRNA maturation *in vitro* and *in vivo* and block the progression of pancreatic cancer cell lines with *TP53* mutations or p53 knockdown, while there was no significant effect on the pancreatic cancer cell lines with high p53 expression. Our study is the first to explore the effectiveness of STRAP in pancreatic cancer, providing a new target for the treatment of patients with poor prognosis in PDAC mainly caused by *TP53* mutation.

Taken together, our study identified the key contribution factor *TP53* that influenced the prognosis of PDAC based on a large sample analysis of public databases. In addition, we found a possible mechanism for disease progression in *TP53* mutant PDAC patients, and uncovered a new effective potential therapeutic target that can interfere with this pathway (**Supplementary Figure 5**). Our research provides reliable theoretical basis for precise classification and clinical adjuvant treatment of pancreatic cancer patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Wenzhou Medical University.

AUTHOR CONTRIBUTIONS

SH, XC, and QZ contributed to conception and design of the study. SH, XC, and XX organized the database. SH, XC, XX, CZ, WH, YZ, PA, HY, KS, and BC performed the experiments and the statistical analysis. SH, XC, PA, and QZ wrote the first draft of the manuscript. XX, CZ, WH, YZ, HY, KS, and BC wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.594224/full#supplementary-material

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Heterogeneity of *TP53* Mutations and P53 Protein Residual Function in Cancer: Does It Matter?

Paola Monti[1], *Paola Menichini*[1], *Andrea Speciale*[1], *Giovanna Cutrona*[2], *Franco Fais*[2,3], *Elisa Taiana*[4,5], *Antonino Neri*[4,5], *Riccardo Bomben*[6], *Massimo Gentile*[7], *Valter Gattei*[6], *Manlio Ferrarini*[3], *Fortunato Morabito*[8,9] *and Gilberto Fronza*[1]*

[1] Mutagenesis and Cancer Prevention Unit, Istituto di Ricerca e Cura a Carattere Scientifico (IRCCS) Ospedale Policlinico San Martino, Genoa, Italy, [2] Molecular Pathology Unit, IRCCS Ospedale Policlinico San Martino, Genoa, Italy, [3] Department of Experimental Medicine, University of Genoa, Genoa, Italy, [4] Department of Oncology and Hemato-oncology, University of Milan, Milan, Italy, [5] Hematology, Fondazione Cà Granda IRCCS Policlinico, Milan, Italy, [6] Clinical and Experimental Onco-Haematology Unit, Centro di Riferimento Oncologico, I.R.C.C.S., Aviano, Italy, [7] Hematology Unit, Azienda Ospedaliera (AO) di Cosenza, Cosenza, Italy, [8] Unità di Ricerca Biotecnologica, Azienda Sanitaria Provinciale di Cosenza, Aprigliano, Italy, [9] Department of Hematology and Bone Marrow Transplant Unit, Augusta Victoria Hospital, Jerusalem, Israel

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***Correspondence:**
Gilberto Fronza
gilberto.fronza@hsanmartino.it

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The human *TP53* locus, located on the short arm of chromosome 17, encodes a tumour suppressor protein which functions as a tetrameric transcription factor capable of regulating the expression of a plethora of target genes involved in cell cycle arrest, apoptosis, DNA repair, autophagy, and metabolism regulation. *TP53* is the most commonly mutated gene in human cancer cells and *TP53* germ-line mutations are responsible for the cancer-prone Li-Fraumeni syndrome. When mutated, the *TP53* gene generally presents missense mutations, which can be distributed throughout the coding sequence, although they are found most frequently in the central DNA binding domain of the protein. *TP53* mutations represent an important prognostic and predictive marker in cancer. The presence of a *TP53* mutation does not necessarily imply a complete P53 inactivation; in fact, mutant P53 proteins are classified based on the effects on P53 protein function. Different models have been used to explore these never-ending facets of *TP53* mutations, generating abundant experimental data on their functional impact. Here, we briefly review the studies analysing the consequences of *TP53* mutations on P53 protein function and their possible implications for clinical outcome. The focus shall be on Chronic Lymphocytic Leukemia (CLL), which also has generated considerable discussion on the role of *TP53* mutations for therapy decisions.

Keywords: *TP53* mutations, chronic lymphocytic leukemia, clinical impact, P53 protein function, reactivation of P53

INTRODUCTION

The human tumour suppressor gene *TP53*, located at 17p13.1 locus, encodes a 393 amino acid-long protein, which was discovered in the 80s of last Century within a complex containing the viral SV40 large T antigen (1–3). Initially misclassified as an oncogene, because of the isolation of mutant cDNA clones capable of inducing cell transformation, the wild type (WT) *TP53* gene was eventually classified as

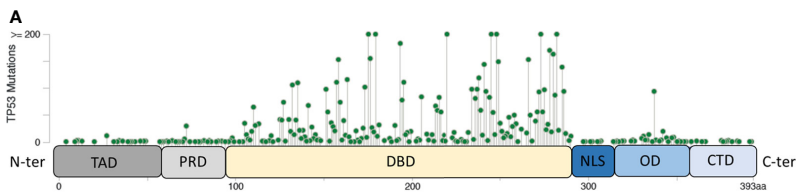
tumour suppressor, upon the definite demonstration of its capacity of inhibiting the growth and the oncogenic transformation of cells in culture (4). Concurrently, somatic *TP53* mutations were identified in tumours (5, 6) and germ-line *TP53* mutations were described in the Li-Fraumeni syndrome (LFS), the well-known hereditary cancer predisposition disorder (7).

The P53 protein consists of different functional domains including mainly a N-terminal transactivation domain (residues 1–61, TAD), a central DNA binding domain (residues 94–290, DBD), an oligomerization domain (residues 325–356, OD) and a C-terminal domain that regulates the DNA binding (residues 357–393, CTD) (**Figure 1A**) (10). While the TAD domain interacts with components of the transcription machinery, the OD and the DBD domains are necessary for the formation of the P53 tetramer which interacts with specific DNA target sequences, called P53 response elements (P53 REs) that are comprised of two degenerate decameric sequences [Pu (Purine)-Pu-Pu-C-A/T-AT-G-Py (Pyrimidine)-Py-Py] separated by a variable spacer (11). To complicate the scenario further, different isoforms of P53, resulting from the usage of alternative promoters and splicing sites, or alternative initiation sites of translation, have been recently described (12, 13).

P53 is a transcription factor (TF) which can be induced by endogenous and exogenous stresses (e.g. oncogenes and UV radiation); these stresses activate signals, which operate *via* post-translational modifications on P53 protein (e.g. phosphorylations, acetylations), and cause release of P53 from the mortal embrace with MDM2 protein, eventually leading to P53 activation (14). P53 protein can then enter the nucleus where it induces the expression of a plethora of target genes (15). However, increasing observations are reporting certain "non-transcriptional functions" of P53, that can contribute to tumour suppression activity (16).

TP53 MUTATIONS HETEROGENEITY

Unlike other tumour suppressors, the *TP53* gene is mostly altered by missense mutations, mainly involving the portion coding for the DBD domain of the P53 protein. Within this region, eight amino-acid substitutions (i.e. R175H, G245S, R248Q, R248W, R249S, R273H, R273S, and R282W), called hotspot mutations, characterize ~27% of all mutant P53 proteins identified in human cancers. However, the spectrum



B

ACTIVITY	MUTATIONS
Loss of function	e.g.: R175H, G245S, R248Q, R248W, S241F, R249S, R273C, R273H, C275Y, R280K
Partial function and/or Temperature sensitive	e.g.: A161T, R181L, R202S, Y220H, S215C, D228V, V272L, R282W
Wild type-like or Super-transactivating	e.g.: T123A, G199H, S240N, S288K, R337H, G360V
Altered specificity	e.g.: K120R, S121F, V122A, T125R, G279E
Dominant	e.g.: R175H, G245S, R248Q, R248W, S241F, R249S, R273C, R273H, C275Y, R280K
Gain of function	e.g.: R175H, G245S, R248Q, R248W, S241F, R249S, R273C, R273H, C275Y, R280K, D281G, R282W

FIGURE 1 | **(A)** Domain organization of P53 protein (TAD, transactivation domain; PRD, proline-rich domain; DBD, DNA-binding domain; NLS, nuclear localization signal; OD, oligomerization domain; CTD, C-terminal domain) and distribution of *TP53* missense mutations from the cBioPortal online tool (TCGA) (8, 9). Missense mutations location (green signs) throughout the P53 protein is shown according to the frequency and the position of the amino acid hit. **(B)** Heterogeneity of mutants P53 with examples of the corresponding *TP53* mutations.

of missense mutations is extremely broad (**Figure 1A**) (8, 9, 17, 18) and varies in the different tumour types.

Mutant P53 proteins have been primarily categorized as "contact" or "structural", depending on whether substituted amino-acid was directly involved in the interaction with DNA (e.g. R273H) or caused a global effect on the protein structure (*e.g.* R175H) (19, 20). Over the last 25 years, a large amount of experimental data on the functional impact of different amino-acid substitutions has been generated and different models have been used to explore the never-ending facets of the corresponding mutant proteins. One of these models is the yeast *S. cerevisiae*, exploiting the fact that P53 can act as TF also in this organism by binding P53 REs located upstream a reporter gene (21, 22). The assay, originated as a Functional Analysis of Separate Alleles in Yeast to study the *TP53* status in somatic tumour and blood cells, has been unceasingly modified and upgraded in order to explore additional functions of P53 mutants (23–26). In 2003 Ishioka's group tested the functionality of more than 2,000 different P53 single amino-acid substitutions expressed in yeast (27). Their work, along with that of others (23–26), led to a re-classification of the *TP53* mutations based on their effects on P53 function generating the following categories: i) loss of function (LOF), ii) partial function and/or temperature sensitive (PF, TS), iii) WT-like or super-transactivating (WT-L, ST), iv) with altered specificity (AS) (i.e. active or partially active on some targets but inactive on others), and v) dominant-negative (DN), based on the ability to inhibit WT protein in a heterozygous condition. However, mutant P53 proteins might be classified also as gain of function (GOF) related to the acquisition of novel oncogenic activities, not shared with the WT protein (**Figure 1B**). This latter property is mainly based on the ability of the mutant P53 to interact with other TFs or with chromatin-modifying complexes, altering the cellular transcriptional profile (28–32). Therefore, mutant P53 proteins appear to constitute a functional rainbow (**Figure 1B**) (33).

Recently, two seminal papers confirmed the relevance of the heterogeneity of mutants P53 in terms of Relative Fitness Score (RFS) in *in vitro* cultures (34, 35). RFS has been used as an indicator of the functional impact of *TP53* mutations in terms of selective growth; specifically, a high RFS indicates a higher fitness of the *TP53* variant with preferential expansion within mixed cultured cells in competition with other *TP53* variants, whereas a low RFS pinpoints preferential depletion in the same experimental condition. Moreover, Kotler et al. (34) showed that the loss of the anti-proliferative function of WT P53 largely correlates with the occurrence of cancer-associated *TP53* mutations, and that selective GOF properties may further favour specific mutants P53 *in vivo*. An enhanced cellular fitness was also confirmed in association with the loss of WT P53 function or the DN effect associated to specific *TP53* mutations (35).

IMPACT OF *TP53* MUTATIONS HETEROGENEITY: FROM MICE TO LFS CLINICAL CONDITION

A clear evidence of the different impact of *TP53* mutations at the organism level came from the studies with *Trp53* knock-in mice. In mice, the introduction of the R172H mutation (corresponding to the human R175H hotspot) at the germ-line level generates a tumor phenotype similar to that observed in *Trp53* null mice, but with a much higher rate of metastasis (36). The GOF activity of R172H mutation was demonstrated to be associated with the functional inactivation of P63 and P73 TFs (37). In contrast, the R172P mutation in mice (corresponding to the human R175P, PF mutation) caused a delayed tumorigenesis rate with absence of chromosomal instability (38). These findings have been paralleled by observations made in patients affected by LFS, showing that the age of first tumor onset and the spectrum of observed tumors are dependent on the type of *TP53* mutation (39).

The associations of the genotype with clinical outcome was explored in carriers of *TP53* germ-line mutations also using a functional classification of the mutant P53 based on the quantification of their transactivation potential and DN effect in a yeast reporter assay. The analyses revealed that P53 proteins severely deficient in transactivation capability were more frequently associated with more severe cancer proneness syndromes (e.g. LFS) (40), whereas a further classification of these alleles, based on DN effects, did not distinguish clinical subclasses (41).

IMPACT OF *TP53* MUTATIONS HETEROGENEITY IN SOMATIC CANCERS

Although the majority of the studies on the prognostic and predictive role of *TP53* status in human cancers distinguish between patients harboring WT *versus* mutant proteins, some evidence favors a categorization of *TP53* mutations since different mutant P53 proteins can have different biologic effects. Poeta et al. (42) proposed the distinction between "disruptive" and "non-disruptive" *TP53* mutations; while disruptive mutations likely lead to a complete loss of activity of the P53 protein, non-disruptive mutants can encode proteins which retain some of the original functions. This classification was used to stratify patients with *TP53* mutations in head and neck squamous cell carcinoma (42), advanced Non-Small Cell Lung Cancer (43), breast and ovarian tumors (44, 45) and esophageal squamous cell carcinoma (46). However, the association between the type of *TP53* mutations and prognosis was significantly variable in the different cancers.

Recently, Dutta et al. (47) analyzed data from 1,537 patients with Acute Myeloid Leukemia (AML) in order to determine a correlation between *TP53* mutations and clinical outcome. *TP53* mutations have been classified according to (i) their impact on protein structure (disruptive *versus* non-disruptive), (ii) an evolutionary action score that takes into account the evolutionary sensitivity to sequence variation and amino-acid conservation (48) and (iii) the RFS (34). Only the RFS was capable of distinguishing among AML patients with a significantly different overall survival and event-free survival. All these observations complicate the scenario and the definition of the events which are drivers of the disease pathogenesis.

IMPACT OF *TP53* MUTATIONS HETEROGENEITY: RESTORATION OF WT P53 FUNCTIONALITY

Mutant P53 protein has been considered a promising target for the development of new anticancer strategies and, in the last two decades, several molecules have been developed with the aim of reinstating the WT function or eliminating the mutant P53 accumulated in cancer cells (49). Upon re-acquisition of its original WT properties, the P53 protein should become transactivation competent (50), and trigger an efficient apoptotic response following treatments to which the tumor cells are normally refractory. PRIMA-1 and its methylated derivative PRIMA-1^{Met} (also named APR246) are the most widely studied molecules on which phase I/II clinical trials are in progress (51). These molecules were found capable of reconstituting the specific DNA binding capacities to different mutant proteins (e.g. R273H, R175H) and of inducing significant apoptosis in cancer cells carrying a mutant P53 protein (52). Another molecule, RITA, which interacts with P53 and inhibits its binding to MDM2, induces a P53-dependent gene transcription and cell death (53). Beside these, many others molecules, which target the interaction of WT P53 with negative regulators (e.g. Nutlins) or with the mutant P53 (e.g. CP31398), have been investigated, some of them being currently tested in clinical trials (53, 54).

A different approach is based on the potential inhibition of the GOF activities, obtained by promoting mutant P53 protein degradation. Since mutant P53 is stabilized by the heat shock protein HSP90, usually over-expressed in cancer cells (55), several HSP90 inhibitors, such as 17-AAG or Ganetespib, have been tested as anticancer molecules and their ability to trigger mutant P53 degradation has been demonstrated (56). Also Histone Deacetylase inhibitors (HDAC), such as SAHA, can induce the degradation of the mutant P53, restraining tumor growth *in vivo* (56, 57). Lastly, a role of autophagy to trigger mutant, but not WT, P53 deprivation has been shown in different cancer cells (58–60), identifying the modulation of autophagy as an emerging strategy for cancer therapy (61, 62).

NOT JUST A QUESTION OF *TP53* MUTATIONS

P53 total inactivation in human cancer cells is frequently caused by the alterations of both alleles, comprising the allelic loss due to deletion of the short arm of the chromosome 17 [del(17p)], and the concomitant mutation of the other allele. It is of note that Donehower et al. (63) performing a comprehensive assessment of the P53 pathway involvement in 32 cancers from The Cancer Genome Atlas, demonstrated the loss of the second allele in 91% of the cases with *TP53* mutations. In addition, in heterozygous murine tumours carrying the hotspot GOF allele R248Q, the loss of the remaining WT *TP53* allele was a necessary prerequisite for the stabilization of the mutant P53 and for the GOF properties to become evident *in vivo* (64). These observations suggest that a given *TP53* mutation must operate in a specific cellular context to show its biological consequences (65).

CLINICAL IMPACT OF *TP53* ALTERATIONS: THE EXAMPLE OF CHRONIC LYMPHOCYTIC LEUKEMIA

Chronic Lymphocytic Leukemia (CLL) is the most common leukemia in the Western countries, characterized by the clonal expansion of CD5+ B cells in peripheral blood, lymph-nodes and bone marrow. CLL clinical course is highly heterogeneous (66), ranging from decades of survival with no need for treatment, to a rapid disease progression with the requirement for an early treatment (67). Such a scenario likely reflects the cellular and molecular heterogeneity of the disease. CLL cases present specific karyotype aberrations, the most frequent being 13q- (~55%), 11q- (~15%) 17p- (~8%) and +12 (~15%), which correlate with a different disease course and outcome (68). In addition, gene mutations (e.g. *TP53*, *SF3B1*, *BIRC3*, and *NOTCH1*) have been reported (69), which, again, may influence the disease course and outcome. B cell receptors (BCR) features expressed by the leukemic cells also dictate the subsequent patient fate as demonstrated by the fact that patients with somatically mutated IGHV genes in the leukemic cells have a better clinical course and outcome than the patients in whom such genes are not somatically mutated; it is generally assumed that stimulation of the leukemic cells by self or exogenous antigens may promote clonal expansion (70). This notion is supported by the observation that inhibitors of the BCR-dependent signal transducing pathway are efficient treatments for CLL (70). In addition, different CLL patients that share the same BCR have similar clinical courses (71). Finally, patients with complex karyotypes, detected by chromosome G-banding, may have a dire prognosis, even in the era of new drugs (72).

P53 dysfunction has certainly a role in the clinical evolution of CLL (73). The incidence of *TP53* mutations is low at diagnosis (<10% of patients), although it rises in cases with progressive disease and reaches approximately 40% in refractory CLL (73–77). Furthermore, there is evidence that CLL patients with *TP53* dysfunction [measured as del(17p) and/or *TP53* mutations] progress more rapidly to stages requiring treatment. Together, these considerations indicate that *TP53* alterations facilitate clonal expansion and disease progression irrespective of the impact they may have on therapy (78). The presence of a P53 dysfunction has a definite negative impact on the effect of chemo-immunotherapy, whereas such impact appears to be less pronounced in patients treated with BCR inhibitors (e.g. Ibrutinib or Idelalisib) or with apoptosis inducers (e.g. Venetoclax). Because of this, *TP53* mutational screening for all patients before therapy start is recommended by the European Research Initiative on CLL group (ERIC) to avoid treatment protocols that are ineffective in patients with P53 dysfunction (79).

Detection of a del(17p) or of a *TP53* mutation is generally assumed to be a sufficient indication for a P53 dysfunction. CLL patients with del(17p) carry a *TP53* mutation in 80% to 90% of

the cases, and ~60% of patients with *TP53* mutations also harbor del(17p), as detected by FISH. Even in the absence of del(17p), the presence of a *TP53* mutation appears to be more frequent in patients with a poor prognosis and a higher genetic complexity (80, 81). Moreover, CLL sub-clones carrying specific *TP53* mutations can be positively selected upon treatment, ultimately becoming the prevalent expansion of an initially minor mutant component (69, 82–84).

DEALING WITH COMPLEXITY IN CLL

The identification of molecular biomarkers together with certain clinical features of the disease may dictate the choice of treatment in CLL (85). Since a P53 dysfunction is the strongest predictor of chemo-refractoriness, the assessment of *TP53* status is the first, and possibly most important, decisional node in the first-line treatment algorithm. Indeed, the presence of P53 dysfunctions prevents the use of chemo-immunotherapy in favour of BCR inhibitors or Venetoclax (78). However, although such drugs have improved the poor efficacy of chemo-immunotherapy in patients with del(17p) and/or *TP53* mutations (86), all these treatments still pose some challenges in these patients. Furthermore, the real influence of a gene dosage effect [e.g. presence of del(17p) *versus* presence of both deletion and a TP53 mutation] in patients treated with the new drugs has still to be clarified (78).

Although genomic technologies are changing the practice of onco-haematology, with improved detection of driver lesions, genomic data, generated through different technologies, each with its own sensitivity, are often considered not only interchangeable [i.e. equivalence between the presence of del (17p) and of a *TP53* mutation (*TP53*mut)], but are also subjected to oversimplification [i.e. equivalence between the presence of one *TP53* alteration (mutation or deletion) and of both alterations)]. Even though a binary simplification (P53 dysfunction *versus* no P53 dysfunction) can be considered clinically usable, the actual situation is potentially more complicated than estimated (87) and a more realistic situation diverging from a simple binary scenario (noDel/noMut *versus* Del and/or Mut) could be conceived and proposed for the clinical use in the future (**Figure 2**). Furthermore, the abundance of the single *TP53* alteration within the leukemic clone [i.e. Variant Allele Frequency (VAF) for a *TP53* mutation and percentage of del(17p) positive cells] may represent a factor of relevance. For example, while all identified *TP53* mutations were clonal with the Sanger sequencing method (VAF>10%), both clonal (VAF>10%) and sub-clonal (VAF<10%, as small as 0.3%) *TP53* mutations can be detected with the introduction of Next Generation Sequencing technologies. Nevertheless, this information has not entered into clinical practice yet, although it may contribute to provide information on the effective P53 function in the leukemic clone and also on its potential prospective evolution. The last update of the guidelines released by ERIC still consider that clinical decisions should be taken based on the presence of a clonal *TP53* mutation.

The percentage of del(17p) positive cell may also represents an important variable as it appears that, among patients with del (17p), those with a higher percentage of cells carrying the deletion have shorter survivals (88). Another layer of

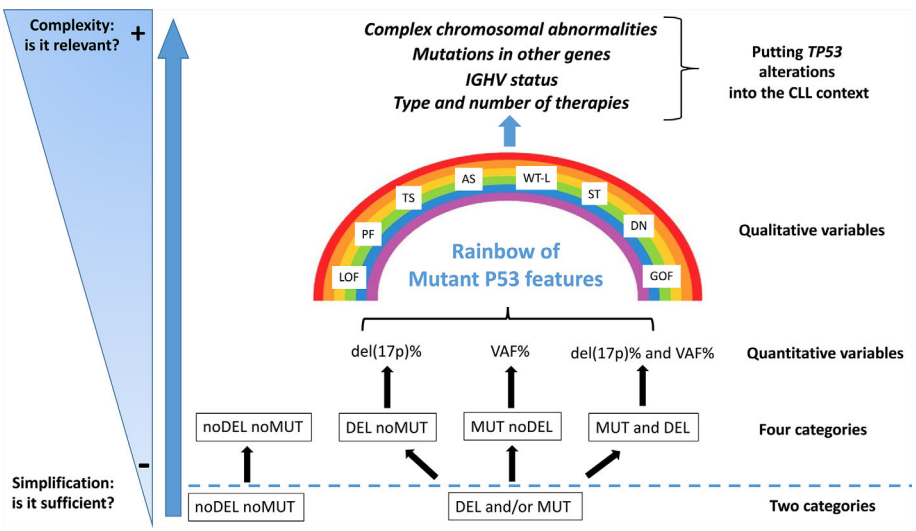


FIGURE 2 | Del(17p) (DEL), detected by FISH, and/or *TP53* mutation (s) (MUT), detected by DNA sequencing, as indicators of a P53 dysfunction. The present classification may represent an oversimplification of the conditions leading to an impaired P53 function, both in terms of quantity [del(17p) % or VAF, Variant Allele Frequency %] and quality (i.e. P53 mutant protein functionality: LOF, loss of function; PF, partial function; AS, altered specificity; TS, temperature sensitive; WT-L, wild-type like; ST, super-transactivating; GOF, gain of function; DN, dominant negative). Moreover, other molecular features (e.g. mutated IGHV *versus* un-mutated IGHV; SF3B1, BIRC3 and NOTCH1 mutations; 13q and 11q partial deletions) and the eventual previous patient management might influence the highly heterogeneous clinical course of the disease.

complexity is related to the fact that not all mutant P53 proteins appear to have the same functional consequences, as previously described. Although these issues, which are related to the effective P53 function in a leukemic clone, have emerged as real biological and clinical problems, they have not been so far deeply investigated in CLL (65). Finally, it should be stressed that *TP53* alterations, although important, are not the sole alterations and should be considered together with other cytogenetic abnormalities which may occur concomitantly in the single patients and may affect *per se* the clinical course of CLL (**Figure 2**).

CONCLUDING REMARKS

In conclusion, is the binary scenario compatible with the underlying complexity in CLL? While a simplified vision is important for deciding clinical strategies, new studies appear necessary for assessing whether further levels of complexity in CLL classification, can lead to a more precise patient stratification. In this context, it is likely that future studies will define whether patients with del(17p) and a *TP53* mutation might have a different clinical course from those who have only a *TP53* mutation or only del(17p). Furthermore, a patient with a partial function *TP53* mutation might show a different clinical course from those harbouring a complete loss of function *TP53* mutation, as suggested by our present observations and studies in other experimental systems (33, 89, 90). All of these aspects regarding P53 dysfunction may affect therapy and consequently deserve an evaluation, possibly more extended than that currently used.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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Beyond the Mevalonate Pathway: Control of Post-Prenylation Processing by Mutant p53

Carla M Borini Etichetti[1], *Evelyn Arel Zalazar*[2†], *Nabila Cocordano*[2†] *and Javier Girardini*[2*]

[1] Instituto de Fisiología Experimental de Rosario, IFISE, CONICET-UNR, Rosario, Argentina, [2] Instituto de Inmunología Clínica y Experimental de Rosario, IDICER, CONICET-UNR, Rosario, Argentina

Missense mutations in the *TP53* gene are among the most frequent alterations in human cancer. Consequently, many tumors show high expression of p53 point mutants, which may acquire novel activities that contribute to develop aggressive tumors. An unexpected aspect of mutant p53 function was uncovered by showing that some mutants can increase the malignant phenotype of tumor cells through alteration of the mevalonate pathway. Among metabolites generated through this pathway, isoprenoids are of particular interest, since they participate in a complex process of posttranslational modification known as prenylation. Recent evidence proposes that mutant p53 also enhances this process through transcriptional activation of *ICMT*, the gene encoding the methyl transferase responsible for the last step of protein prenylation. In this way, mutant p53 may act at different levels to promote prenylation of key proteins in tumorigenesis, including several members of the RAS and RHO families. Instead, wild type p53 acts in the opposite way, downregulating mevalonate pathway genes and *ICMT*. This oncogenic circuit also allows to establish potential connections with other metabolic pathways. The demand of acetyl-CoA for the mevalonate pathway may pose limitations in cell metabolism. Likewise, the dependence on S-adenosyl methionine for carboxymethylation, may expose cells to methionine stress. The involvement of protein prenylation in tumor progression offers a novel perspective to understand the antitumoral effects of mevalonate pathway inhibitors, such as statins, and to explore novel therapeutic strategies.

Keywords: metastasis, carboxymethylation, actin cytoskeleton, CAAX proteins, cancer, methionine restriction, methionine stress

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****Correspondence:***
Javier Girardini
girardini@idicer-conicet.gob.ar

[†]These authors have contributed equally to this work

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INTRODUCTION

TP53, the gene encoding the tumor suppressor p53, is one of the most frequently mutated genes in human cancer (1). More than 70% of *TP53* alterations are missense mutations, leading to the conspicuous presence of p53 point mutants in tumors (2). Mounting evidence has supported the notion that these mutants cooperate with tumorigenesis though the acquisition of novel activities (3). Particularly, animal models provided compelling proof of the ability of p53 point mutants to promote the development of aggressive tumors. Intense research on the mechanisms underlying this

effect has revealed a complex scenario (4). Mutant p53 can be considered as a pleiotropic factor that affects cell behavior by altering the function of different interactors. In this context, the presence of specific arrays of interactors combined with patterns of active signaling pathways may explain the manifold activities described for p53 mutants (5). Most mutations are found in the DNA Binding Domain, and a few codons concentrate the highest mutation frequencies. Although the development of aggressive tumors appears as a common biological outcome of most p53 mutants, some differences were also reported (6). The ability to cooperate with oncogenic mechanisms and the exclusive presence in tumor cells make mutant p53 an attractive therapeutic target. Therefore, much effort is concentrated in the study of its function. In this regard, the unexpected finding that mutant p53 alters the expression of mevalonate (MVA) pathway genes (7) opened new avenues to understand the importance of metabolism in tumor cell biology.

TAKING CONTROL OF THE MEVALONATE PATHWAY, MUTANT VS WILD TYPE P53

The pathological role of alterations on the MVA pathway was initially proposed based on the observation that inhibitors of the enzyme that catalyzes the rate-limiting step (3-hydroxy-3-methyl-glutaryl-CoA reductase, HMGCR), known as statins, reduced the proliferation of tumor cells (8, 9). This pathway allows the biosynthesis of cholesterol and isoprenoids from acetyl-CoA (**Figure 1**). The isoprenoid intermediates farnesyl and geranylgeranyl may be covalently attached to cysteine residues on the carboxyl terminus of proteins, in the first step of the protein prenylation pathway, a complex mechanism of posttranslational modification (10). The connection between mutant p53 and the MVA pathway was unveiled following the observation that several p53 point mutants promoted an aggressive phenotype in three-dimensional (3D) cultures of breast cancer cells (7). The finding that endogenous p53R273H enhanced the expression of at least 17 MVA pathway genes, along with evidence from elegant pharmacologic manipulation of the pathway, led to propose that enhanced flux through the MVA pathway was responsible for the phenotype associated to mutant p53. The expression of MVA pathway genes is under control of Sterol Responsive Element Binding Proteins (SREBPs), which induce transcription in response to low cholesterol levels (11). The recruitment of mutant p53 on the promoters of MVA pathway genes in the vicinity of Sterol Responsive Elements (SREs) as well as the ability of p53R273H to interact with SREBPs suggest that mutant p53 acts as a transcriptional co-activator. Supporting the idea that MVA pathway alteration cooperates with tumor progression, high expression of MVA pathway genes was correlated with poor clinical outcome in breast cancer patients (7).

Protein geranylgeranylation appears to be crucial in the effect of mutant p53. Inhibition of geranylgeranyl transferase I (GGTaseI) attenuated the invasive morphology of MDA-MB-231 cells in 3D cultures, similar to endogenous mutant p53 downregulation. In contrast, inhibition of enzymes that derive the flux of the pathway to other molecules, such as squalene synthase (SQS) and farnesyl transferase (FTase), had no effect. Moreover, addition of geranylgeranyl diphosphate (GGPP) recovered the invasive morphology in cells where mutant p53 was downregulated (7). Furthermore, mutant p53 depletion or HMGCR inhibition by statins reduced the nuclear localization and activity of Yes-Associated Protein (YAP) and Transcriptional coactivator with PDZ-binding motif (TAZ) (12), the transcriptional module of the Hippo pathway, through a mechanism that involves Ras homolog family member A (RHOA) prenylation. Hyperactivation of YAP and TAZ has been increasingly associated to proliferation and metastasis (13). Similarly, YAP/TAZ inactivation was not observed upon inhibition of SQS and FTase, but was phenocopied by GGTaseI inhibition. Moreover, adding GGPP reverted the effect of upstream inhibition of the MVA pathway (12).

The finding that wild type p53 (wt p53) repressed the expression of MVA pathway genes provides strong support to the idea that alteration of this pathway may be a critical event in tumor progression. In this case, an indirect mechanism was described, involving inhibition of SREBP-2 maturation (14). This effect was mediated by the transcriptional induction by wt p53 of ATP binding cassette subfamily A member 1 (*ABCA1)*, which encodes a protein involved in the retrograde transport of cholesterol from the plasma membrane to the endoplasmic reticulum (ER). SREBPs are produced as inactive precursors anchored to the cytosolic side of the ER. Maturation can be stimulated by low cholesterol levels in the ER, which triggers a complex process that leads to proteolytic cleavage and nuclear import of SREBPs (15, 16). Analysis of cancer databases showed that *ABCA1* expression was lower in colon, breast and liver carcinomas comparing with normal tissues. Likewise, *Abca1* inactivation enhanced tumorigenesis in an experimental model of hepatocellular carcinoma (HCC). Additional evidence from animal models strongly supports the notion that ABCA1 is relevant for the tumor suppressive function of wt p53 (14). Noteworthy, wt p53 was also reported to repress the expression of SREBP1-c, suggesting that the interplay between the p53 pathway and SREBPs is even more complex (17, 18).

POST PRENYLATION PROCESSING AND THE P53 PATHWAY

The posttranslational processing pathway known as prenylation involves three stages (**Figure 1**). First, the addition of farnesyl or geranylgeranyl, to a cysteine residue close to the carboxyl terminus of proteins (19), catalyzed by FTase or GGTaseI, respectively. The prenylated cysteine is typically part of a CAAX motif (C: cysteine; A: aliphatic amino acid; X: any amino acid), although other motifs such as CXC can also be targeted (20). Second, the terminal amino acids following the prenylated cysteine are removed by the specific peptidase RAS Converting Endoprotease 1 (RCE1) in the ER (21). Third, Isoprenylcysteine Carboxyl Methyltransferase (ICMT), also an integral membrane protein of the ER, catalyzes the methylation

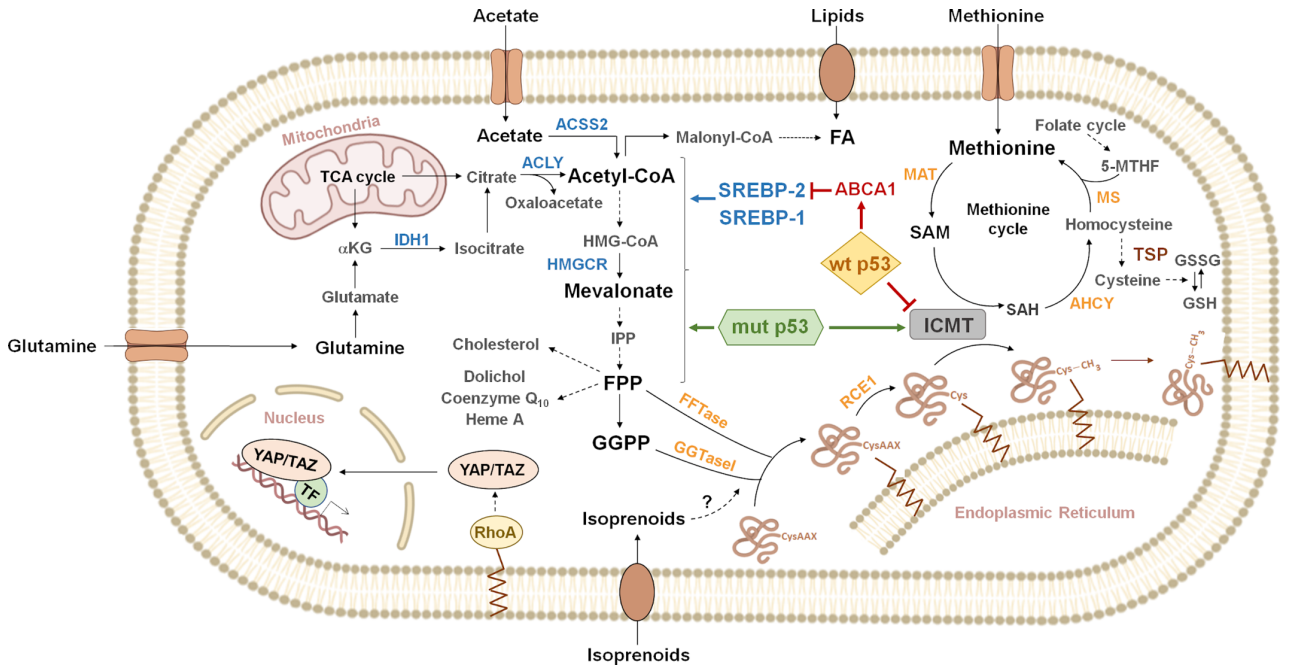

FIGURE 1 | Overview of metabolic pathways connected to protein prenylation. Citrate generated from glutamine or the tricarboxylic acid cycle (TCA cycle) is cleaved by ATP-citrate lyase (ACLY) to acetyl-CoA and oxaloacetate. Acetyl-CoA can also be synthesized by cytoplasmic acetyl-CoA synthetase (ACSS2) from exogenous acetate. Acetyl-CoA, can be carboxylated to malonyl-CoA, to produce fatty acids (FA). Lipids can also be incorporated through exogenous uptake. Alternatively, acetyl-CoA enters the mevalonate pathway, where three molecules are condensed in a two-step reaction to produce 3-hydroxy-3-methylglutaryl CoA (HMG-CoA). HMG-CoA is then reduced by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) to produce mevalonate. Mevalonate is then converted into isopentenyl-diphosphate (IPP) through a series of enzymatic steps. IPP serves as a monomeric unit for the consequent synthesis of farnesyl diphosphate (FPP), geranylgeranyl diphosphate (GGPP) and other downstream metabolites (cholesterol, dolichol, coenzyme Q10, heme A, etc.). The isoprenoid moiety of FPP or GGPP may be covalently attached to cysteine residues on the carboxyl terminus of some proteins, in the first step of the protein prenylation pathway. For example, the activity of RHOA is regulated by geranylgeranylation, which localizes RHOA to the plasma membrane. RHOA promotes the nuclear localization and activity of the Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ). Gain of function p53 mutants (mut p53) functionally interact with sterol regulatory element binding proteins (SREBPs) to drive increased expression of mevalonate pathway genes. In contrast, wild type p53 (wt p53) represses the mevalonate pathway genes through inhibition of SREBP-2 maturation, as a consequence of transcriptional induction of ATP binding cassette subfamily A member 1 (*ABCA1*). Additionally, p53 point mutants can induce isoprenylcysteine carboxymethyltransferase (ICMT) expression, while wt p53 exerts the opposite effect, through transcriptional repression. ICMT catalyzes protein carboxymethylation, the last step of the protein prenylation pathway. The methyl donor in this reaction is S-adenosyl methionine (SAM), which is produced from the essential aminoacid methionine in the rate-limiting reaction catalyzed by methionine adenosyl transferase (MAT). SAM is transformed into S-adenosyl homocysteine (SAH), which can be used to regenerate methionine through the methionine cycle. Homocysteine can be derived to the transsulfuration pathway (TSP) to synthesize glutathione (GSH). Dashed arrows represent multiple enzymatic steps. Indications on reversibility of enzymatic reactions and subcellular localization of some enzymes have been omitted for simplicity. Enzymes known to be regulated by SREBPs are shown in blue. IDH1, isocitrate dehydrogenase 1; α-KG, α-ketoglutarate; FFTase, farnesyl transferase; GGTaseI, geranylgeranyl transferase 1; RCE1, RAS converting enzyme 1; AHCY, adenosylhomocysteinase; MS, methionine synthase; 5-MTHF, 5-methyltetrahydrofolate; GSSG, oxidized glutathione; TF, transcription factors.

of the free carboxyl terminus on the cysteine. This modification provides an uncharged hydrophobic carboxyl terminus, which increases protein interaction with biological membranes and/or modifies its ability to interact with other proteins (22). Only one member of the ICMT methyltransferase class is encoded in mammalian genomes and lacks homology to other methyltransferases (23). Interestingly, methylation of prenylated proteins is absent in ICMT$^{-/-}$ cells, which indicates that ICMT is the only enzyme able to catalyze this reaction (24). A connection between the p53 pathway and post-prenylation processing was established by showing that wt and mutant p53 regulate *ICMT* expression (25). Several p53 point mutants induced *ICMT* expression in breast, colon, and lung cancer cell lines. This effect was associated to transcriptional activation, since mutant p53 was recruited on the *ICMT* promoter and was able to enhance its activity. Moreover, promoter activation and enhanced endogenous gene expression were observed in p53 null cells, showing that this activity is a novel function acquired by mutants. In contrast, wt p53 was also found on the *ICMT* promoter but repressed promoter activity and reduced mRNA and protein levels. Interestingly, the effects of wt and mutant p53 were shown to depend on different promoter regions, indicating that they act through different mechanisms. This evidence suggests that the acquisition of missense mutations on *TP53* may exert a strong effect on *ICMT* expression by complementary mechanisms. The repressive function of wt p53 may be lost upon mutation of *TP53*, while the presence of point mutants may enhance gene expression by wt p53-independent mechanisms. Underlining the clinical relevance of the connection between the p53 pathway and post-prenylation processing, *ICMT* expression

was found to be significantly reduced in patients classified as wt p53, but was increased in mutant p53 patients (25). The discussed evidence suggests that deregulated expression of ICMT may cooperate with tumor progression. In support to this idea, high ICMT levels were found in hepatocellular carcinoma patients and ICMT overexpression enhanced proliferation and migration in normal liver cells (26). Similarly, ICMT overexpression in H1299 non-small cell lung carcinoma (NSCLC) cells increased clonogenic potential *in vitro* and tumorigenesis in a xenograft model. Moreover, analysis of breast and lung cancer databases showed that high *ICMT* expression was correlated with reduced survival (25).

ICMT TARGETS IN ONCOGENESIS

ICMT substrates are distributed among different families (**Table 1**), complicating the rationalization of its pathological effects. In addition to RAS and RHO families of GTPases, more than 200 CAAX proteins have been predicted based on structural analysis (70, 71). Polypeptides ending in CXC, as the doubly geranylgeranylated RAB family, are also modified by this pathway. The identification of RAS family members as ICMT substrates reinforced the notion that protein prenylation may play a role in cancer (72, 73). Deletion of *Icmt* reduced KRAS-induced transformation *in vitro* (74) and neoplastic lesions in a mouse model of myeloproliferative syndrome (75). Moreover, genetic ablation of *ICMT* in RAS-transformed human breast primary cells and human breast cancer cell lines harboring mutant RAS, reduced tumor formation in xenograft models (76). Intriguingly, *Icmt* inactivation in a KRAS-driven mouse model of pancreatic carcinoma increased the number of pancreatic neoplasias and promoted tumor progression (77). Impairment of Notch signaling through deregulation of RAB7 and RAB8 was suggested as responsible for this effect. Considering the impact of mutant p53 as a promoter of pancreatic cancer (78), It will be interesting to explore the interplay between the MVA pathway and protein prenylation in this pathology.

The deregulated action of ICMT on RHO GTPases may promote invasiveness and metastasis through alteration of cytoskeleton remodelling and cell motility. Accordingly, ICMT inhibition reduced migration and invasion in MDA-MB-231 cells (53), concomitant with decreased RHOA and RAC1 activity. The ability of miR-100 to attenuate lamellipodia formation, matrix metallopeptidase 2 (MMP2) activation and metastasis in hepatocellular carcinoma cells was associated to ICMT-RAC1 signaling inhibition (79). Likewise, reduced migration, invasion and metastasis were observed in HT-1080 fibrosarcoma cells upon ICMT inhibition (80), which was associated to RAB4A impaired function. ICMT overexpression in H1299 cells significantly affected actin cytoskeleton, suggesting an effect on RHO GTPases (25). Interestingly, some evidences reported differential effects of ICMT on subcellular localization and/or expression levels of protein substrates, arguing for a role in the concerted regulation of prenylated proteins. For example, ICMT inhibition reduced RHOA half-life, but enhanced RAS stability (74, 81). Lack of ICMT had different effects on the subcellular localization of RAS and RHO family members (49), and on the localization and stability of RALA and RALB. Dynamic regulation of protein carboxymethylation may have relevant consequences

TABLE 1 | List of prenylated proteins and ICMT substrates.

Protein Name	CAAX motif	Prenyl Group	ICMT substrate	Reference
G protein-coupled receptor kinase 1 (GRK1)	CLVS	15C	Yes	(27, 28)
G protein subunit gamma transducin 1 (GNGT1/GNG1)	CVIS	15C	Yes	(29, 30)
G protein subunit gamma 2 (GNG2)	CAIL	20C	Yes	(29, 30)
Lamin B1 (LMNB1)	CAIM	15C	Yes	(31–33)
Lamin A (LMNA)	CSIM	15C	Yes	(34, 35)
ERAS	CSVA	15C	Yes	(36)
HRAS	CVLS	15C	Yes	(37, 38)
KRAS4A	CIIM	15C	Yes	(24, 37, 38)
KRAS4B	CVIM	15C	Yes	(24, 37, 38)
NRAS	CVVM	15C	Yes	(37, 38)
RAB3B	CSC	20C	Yes	(39)
RAB3D	CSC	20C	Yes	(39)
RAB4A	CGC	20C	Yes	(40, 41)
RAB6A	CSC	20C	Yes	(39)
RAB7A	CSC	20C	Yes	(41)
RAB8A	CVLL	20C	Yes	(41, 42)
RAB13	CSLG	20C	Yes	(41, 42)
RAB18	CSVL	20C	Yes	(41)
RAB23	CSVP	20C	Yes	(41)
RAB27A	CGC	20C	Yes	(41)
RALA	CCIL	20C	Yes	(43–45)
RALB	CCLL	20C	Yes	(44, 45)
RHEB1	CHLM	15C	Yes	(36, 46)
RHEB2	CSVM	15C	Yes	(36, 46)
RHOA	CLVL	20C	Yes	(47, 48)
RHOB	CKVL	15C/20C *	Yes	(49–51)
RHOC	CPIL	20C	Yes	(49, 51)
RHOD	CVVT	15C	Yes	(49)
RHOH	CKIF	15C/20C *	Yes	(49)
CDC42	CCIF	20C	Yes	(37, 52)
RAC1	CLLL	20C	Yes	(53, 54)
RAC2	CSLL	20C	Yes	(37, 43, 55)
RAC3	CTVF	20C	Yes	(54, 56)
Phosphodiesterase 6A (PDE6A/PDEα)	CCIQ	15C	Yes	(28, 57, 58)
Phosphodiesterase 6B (PDE6B/PDEβ)	CCIL	20C	Yes	(28, 57, 58)
Lamin B2 (LMNB2)	CYVM	15C	Methylation	(33, 59)
RAB3A	CAC	20C	Methylation	(20, 60)
RAP1A	CLLL	20C	Methylation	(61)
RAP1B	CQLL	20C	Methylation	(62)
STK11/LKB1	CKQQ	15C	ND	(63)
PTP4A1/PTPCAAX1	CCIQ	15C/20C*	ND	(64)
PTP4A2/PTPCAAX2	CCVQ	15C/20C*	ND	(64)
RAP2A	CNIQ	15C	ND	(65)
RAP2B	CVIL	20C	ND	(65)
RAP2C	CVVQ	20C	ND	(66, 67)
PPP1R16B/TIMAP	CRIS	15C	ND	(68, 69)

The specific CAAX or CXC motifs and the type of isoprenoid (15C farnesyl group; 20C, geranyl-geranyl group), are indicated. Cases where there is experimental evidence on the involvement of ICMT are indicated. Methylation: proteins shown to be carboxymethylated but without evidence on the involvement of ICMT. ND, not determined. (*) Proteins reported to be farnesylated or geranyl-geranylated.

as suggested by the identification of carboxylesterase 1 (CES1), a carboxylesterase affecting the methylation status of RHOA. Interestingly, RHOA activity and cytoskeleton organization in breast cancer cells were similarly affected by *CES1* silencing and ICMT overexpression (82).

ACETYL-COA AND METABOLIC STRESS IN TUMOR CELLS

Availability of acetyl-CoA may be a critical aspect in tumor cells that sustain aggressive phenotypes by exploiting the MVA pathway. Acetyl-CoA is the starting point of the MVA pathway; however, it is also required for other important pathways, as fatty acids (FA) biosynthesis (**Figure 1**). An important source of acetyl-CoA is citrate produced in the mitochondria by the tricarboxylic acid (TCA) cycle, which can be converted in the cytosol into oxaloacetate and acetyl-CoA by ATP citrate-lyase (ACLY) (83). In addition, exogenous acetate may be directly converted into acetyl-CoA by cytoplasmic acetyl-CoA synthetase (ACSS2) (84). Glutamine uptake also allows the indirect production of acetyl-CoA through a series of reactions that take place in the cytosol (85, 86). A strong requirement of acetyl-CoA may expose tumor cells to the dependence on specific metabolic capabilities, forcing cells to shape their metabolism. Accordingly, there is evidence showing enhanced activity of ACLY (87) and ACSS2 (88) in cancer cells, as well as of isocitrate dehydrogenase 1 (IDH1) (89, 90), which catalyzes reductive carboxylation in the conversion of glutamine into acetyl-CoA. Expression of these genes is regulated by SREBPs suggesting the intriguing possibility that they may be induced by mutant p53 and repressed by wt p53 (91–95). Oxygen availability is frequently limited in the tumor microenvironment and nutrient uptake is highly conditioned by the degree of neovascularization (96). Entry of pyruvate into the mitochondria may be inhibited under hypoxic conditions (97), downregulating the TCA cycle and citrate production. Under these conditions, acetate and glutamine as alternative sources of acetyl-CoA may become critical. Moreover, if uptake of exogenous lipids is not able to satisfy the high demand in proliferating cells, active FA biosynthesis may be expected to compete with the MVA pathway for acetyl-CoA. In this scenario, strategies aimed at interfering with alternative acetyl-CoA sources may be effective to counteract cancer cell proliferation.

ICMT LINKS THE MEVALONATE PATHWAY WITH METHIONINE METABOLISM

The methyl donor in protein carboxymethylation is S-adenosyl methionine (SAM), which is produced from the essential aminoacid methionine, in a reaction catalyzed by methionine adenosyl transferase (MAT). SAM is also the methyl donor in other reactions, including methylation of DNA, RNA, non-prenylated proteins and in polyamine biosynthesis. Upon methylation, SAM is transformed into S-adenosyl homocysteine (SAH), which can be used to regenerate methionine through the methionine cycle (98) (**Figure 1**). This cycle is closely interconnected with two other metabolic processes. Hydrolysis of SAH, catalyzed by adenosylhomocysteinase (AHCY), produces homocysteine, which can react with 5-methyl-tetra-hydrofolate (5-MTH) generated in the folate cycle, giving back methionine. Alternatively, homocysteine can be diverted to the transsulfuration pathway that ultimately leads to the synthesis of glutathione (GSH). Alteration of methionine cycle enzymes were related to cancer. For example, *MAT2A* and *MAT2B*, the genes coding for the subunits of the most abundant MAT isoenzyme, were found upregulated in tumors and cancer-initiating cells (99, 100). The close connection between SAM and the one-carbon metabolic network suggests that cell context and nutritional state may affect ICMT activity. Methionine availability may decrease SAM levels, thereby limiting ICMT catalyzed carboxymethylation. Therefore, limiting methionine uptake may have a selective inhibitory effect on cancer cells that benefit from ICMT hyperactivation. Accordingly, pioneering observations reported a marked requirement of methionine on transformed rat and human cells (101). Moreover, dietary methionine restriction reduced tumor growth and metastasis in animal models, and increased sensitivity to chemotherapeutic agents (98). Nevertheless, the molecular mechanisms underlying these effects are not yet clear.

Homocysteine is a key molecule in the one-carbon network, since it connects the methionine cycle with the folate cycle and GSH production. Under strong oxidative stress conditions, high availability of GSH may be required and, therefore, homocysteine may be preferentially driven to the transsulfuration pathway, precluding the possibility to regenerate methionine. Therefore, enhanced ICMT activity in cells under oxidative stress may further increase the dependency on methionine. Moreover, SAH acts as a negative feedback inhibitor of ICMT (102). Treatment with the AHCY inhibitor adenosine dihaldeyde (AdOx) produced accumulation of SAH (103) and reduced *in vitro* invasion and migration of cancer cell lines (104).

DISCUSSION

Several metabolites produced by the MVA pathway may affect cell behavior, however, the positive effect of mutant p53 on the expression of MVA pathway genes and *ICMT* underline the relevance of isoprenoids in cancer. Conversely, the negative regulation exerted by wt p53 on SREBP-2 maturation and *ICMT* expression indicates that MVA pathway and carboxymethylation of prenylated proteins should be strictly regulated under physiological conditions. The concerted effects of mutant p53 on MVA and prenylation pathways allow tumor cells to connect both pathways, thereby fostering full modification of prenylated proteins playing key roles in oncogenesis. Still, selective alteration of each pathway may be enough to promote tumor progression. In this way, mutant p53 may activate alternative mechanisms useful to promote

tumorigenesis in different contexts. Since exogenous isoprenoids may be incorporated into cancer cells and phosphorylated (105), the intriguing possibility that protein prenylation may be exploited by tumors independently from the MVA pathway may also be considered. Noteworthy, exogenous supplementation of geranylgeraniol counteracted the antitumoral effect of pitavastatin in a xenograft model of ovarian cancer cells (106). The correlation of ICMT expression with clinical outcome and the pro-oncogenic effects observed in experimental systems point at ICMT overexpression as a relevant event in tumor progression. Consequently, the potential of ICMT as a therapeutic target encouraged the identification of inhibitors. Isoprenylated cysteine analogs inhibited ICMT activity and showed antiproliferative effects, however, their mechanism of action is not clear since some of them act as modulators of RAS chaperones (107, 108). Indole-based molecules were also proposed, such as Cysmethynil (109), a competitive inhibitor with respect to isoprenylated cysteine and a non-competitive inhibitor with respect to SAM, which showed antitumor activity *in vitro* and *in vivo* (10, 110, 111). In summary, alteration of MVA pathway and protein prenylation by mutant p53 revealed interesting connections to explore. Understanding the role of less studied ICMT substrates in cancer and the study of mechanisms that regulate ICMT activity will be critical to dissect the molecular mechanisms underlying ICMT pathological effects.

AUTHOR CONTRIBUTIONS

JG designed and wrote the manuscript. CBE contributed to manuscript writing and performed the figures. EAZ and NC contributed to the manuscript writing and figures. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.595034/full#supplementary-material

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REVIEW
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Mutant p53 in Cancer Progression and Targeted Therapies

Gaoyang Zhu[1], *Chaoyun Pan*[2], *Jin-Xin Bei*[3], *Bo Li*[2], *Chen Liang*[4], *Yang Xu*[5,6]* *and Xuemei Fu*[5]*

[1] Postdoctoral Research Center, Shunde Hospital, Southern Medical University (The First People's Hospital of Shunde), Foshan, China, [2] Department of Biochemistry and Molecular Biology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China, [3] Department of Experimental Research, Sun Yat-sen University Cancer Center, State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Guangdong Key Laboratory of Nasopharyngeal Carcinoma Diagnosis and Therapy, Guangzhou, China, [4] Shenzhen International Institute for Biomedical Research, Shenzhen, China, [5] Department of Pediatrics, The Eighth Affiliated Hospital, Sun Yat-sen University, Shenzhen, China, [6] Division of Biological Sciences, University of California, San Diego, La Jolla, CA, United States

TP53 is the most frequently mutated tumor suppressor gene in human cancer. The majority of mutations of p53 are missense mutations, leading to the expression of the full length p53 mutant proteins. Mutant p53 (Mutp53) proteins not only lose wild-type p53-dependent tumor suppressive functions, but also frequently acquire oncogenic gain-of-functions (GOF) that promote tumorigenesis. In this review, we summarize the recent advances in our understanding of the oncogenic GOF of mutp53 and the potential therapies targeting mutp53 in human cancers. In particular, we discuss the promising drugs that are currently under clinical trials as well as the emerging therapeutic strategies, including CRISPR/Cas9 based genome edition of mutant *TP53* allele, small peptide mediated restoration of wild-type p53 function, and immunotherapies that directly eliminate mutp53 expressing tumor cells.

Keywords: mutant p53 protein, gain-of-function, targeted therapy, tumorigenesis, drug resistance

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***Correspondence:**
Yang Xu
yangxu@ucsd.edu
Xuemei Fu
fxmzj2004@163.com

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INTRODUCTION

Tumor suppressor p53 is the principal cellular responder to various stress signals such as oncogene activation, DNA damage, hypoxia, reactive oxygen species (ROS), etc. Upon activation, p53 induces numerous cellular responses including cell cycle arrest to restore genetic integrity, or apoptosis, senescence, or ferroptosis to eliminate unrecoverable cells. Therefore, p53 is considered the "Guardian of the genome" to prevent accumulation of oncogenic mutations that lead to malignant tumor (1, 2).

Mutations in *TP53* are found in over half of human cancers, thus is known as the most commonly mutated gene in human cancers (3, 4). Different from many other tumor suppressor genes which generally undergo deletion or truncation in cancer cells, mutations in *TP53* allele are predominantly missense mutations which give rise to a single amino acid substitution in the full-length mutant protein (5). p53 protein is composed of three functional domains including a transactivation and proline rich domain, a central DNA-binding domain (DBD), and an oligomerization domain (6). While mutations can occur spontaneously throughout the p53 gene, the majority of p53 missense mutations are located in the central DBD region of the p53 gene, which binds to its consensus DNA binding sites to regulate its target gene expression. These missense

mutations are divided into two categories: DNA contact mutations such as R248Q and R273H that directly occur at the amino acids mediating p53-DNA interaction, and conformational mutations that indirectly disrupt p53-DNA interaction by inducing local (R249S and G245S) or global (R175H and R282W) conformational changes due to the reduced thermostability caused by these mutations (5). Nearly one third of all p53 mutations occur at these six "hotspot" mutational residues (3).

Mutations in the p53 gene can appear at either the initial-stage or the late-stage during tumorigenesis depending on the origin of cancer types, and strongly facilitate the onset or progression of cancers (7). Functionally, p53 mutants (mutp53) not only lead to the loss of wild-type p53 functions, but can also result in a dominant negative effect by forming hetero-tetramers with the remaining wild-type p53 expressed from the other wild-type allele. p53 mutations are usually followed by the loss of heterozygosity (LOH) at the remaining wild-type *TP53* allele, leading to the complete loss of wild-type p53 in late-stage tumors, and further confer these cancer cells a selective advantage during cancer development (8, 9). Most p53 missense mutants acquire oncogenic gain-of-function (GOF) activities. For example, conformational changes of mutp53 enable them to interact with many transcription factors such as p63, p73, NF-Y, Sp1, ETS1/2, NF-κB, ATM, and SMADs, altering the transcription, cell cycle, apoptosis and metabolism of cancer cells. This changes lead to increased genetic instability, cellular proliferation, metastasis and chemo-/radio-resistance (10). In addition, the new transcriptional targets acquired by mutp53 is another well established GOF mechanism for mutp53 to promote cancer progression (5). Therefore, to compete for survival in a nutrient-deprived and hypoxic environment, the human tumor cells are under stringent selection for the loss of wild-type p53 function and acquirement of p53 mutants that possess GOF to promote the survival of tumor cells.

In this review, we focus on the gain-of-functions of mutp53 in malignant tumor progression and the current strategies targeting mutp53 for personalized therapeutic treatments, aiming to provide insights into targeted treatment of human cancers with p53 mutation.

MUTANT p53 FACILITATES CANCER PROGRESSION

Induction of Genetic Instability

As the "Guardian of the genome", the fundamental goal of WT p53 is to maintain genetic stability by preventing the passage of genetic mutations to daughter cells (1). While p53 null cells still retain certain levels of checkpoint and DNA repair capacities, cells harboring p53 mutant proteins showed a dramatic higher level of genomic instability such as interchromosomal translocations and aneuploidy, indicating the oncogenic GOF activity of p53 mutants (8, 11, 12). These variations largely contribute to genetic diversity that expedites malignant tumor development. Mechanistically, the common p53 mutants can disrupt the earliest stage of DNA double-stranded break (DSB) damage responses by interacting with the nuclease Mre11 to suppress the recruitment of Mre11/Rad50/NBS1 (MRN) complex to the site of DNA DSB damage, leading to inactivation of ATM, the key DNA DSB damage sensor, and the resultant G_2/M checkpoint impairment (8). Mutp53 can also induce genomic abnormality by inactivating DNA replication process. For example, some mutp53 proteins activate cyclin A to promote the formation of DNA replication origin and the intra-S phase checkpoint kinase CHK1 to stabilize the replication forks, facilitating the duplication of aberrant genomic DNAs (13).

Accelerating Proliferation

Accumulating evidence has revealed that mutp53 promotes the limitless replicative potential and insensitivity to anti-growth signals during the malignant transformation of a normal cell, which are two of the key "hallmarks of cancer" (14, 15). Mutp53 was reported to physically interact with the major cell cycle regulator nuclear transcription factor Y (NF-Y), and recruits either acetyltransferase p300 or the main effector of Hippo pathway, YAP, to activate NF-Y target genes including *cyclin A*, *cyclin B*, *cdk1* and *cdc25C* (16, 17). Mutant p53 and YAP have also been found to form another trimeric transcriptional complex with TEAD to induce the expression of circular RNA *circPVT1*, which activates proliferative genes such as *aurka* and *mki67* (18). Mutp53 also regulates the expression of MicroRNA *miR-27a*, which promotes a sustained EGF-induced ERK1/2 activation, thereby facilitating cellular proliferation and tumorigenesis (19). In addition, p53 mutants also target key chromatin regulators including methyltransferases MLL1 and MLL2 and acetyltransferase MOZ, leading to genome-wide increases of active histone modifications H3K4me3 and H3K9ac to enhance proliferation (20). In addition, the *TP53* R249S mutant, frequently detected in HBV positive human hepatocellular carcinoma (HCC), has a unique GOF in regulating proliferation and survival of HCC cells by promoting c-Myc-dependent rDNA transcription essential for ribosomal biogenesis (21).

Modulating Metabolism

Cellular metabolism of glucose, lipid, and nucleotide are the fundamental basis for cell survival, which undergo dramatic changes during malignant transformation. Emerging evidence shows that mutp53 proteins contribute to various aspects of these processes (22, 23). Rapidly dividing tumor cells rely mainly on aerobic glycolysis to meet their high energy and biosynthetic demand, a phenomenon known as Warburg effect (24). Mutp53 has been shown to activate the small GTPase RhoA and its downstream effector ROCK, to promote GLUT1 translocation to the plasma membrane and thus enhance glucose uptake and glycolytic rate (25). Under conditions of energy stress, mutp53 preferentially binds to the AMPKα subunit and directly inhibits the metabolic functions of AMPK signaling, leading to increased aerobic glycolysis as well as lipid production (26). Notably, the roles of mutp53 in promoting lipid metabolism are partly mediated by the mevalonate pathway, which is responsible for *de novo* cholesterol synthesis and generation of many important

nonsterol isoprenoid derivatives. Mutp53 is recruited to the promoters of several mevalonate pathway genes to induce their expression through its interaction with the master transcription factor SREBP1/SREBP2 (27). In addition, the mevalonate pathway-DNAJA1 axis as well as the STAT3-mevalonate pathway axis are both found to prevent mutp53 from being degraded by CHIP ubiquitin ligase, forming a positive-feedback loop to ensure rapid lipid synthesis (28, 29). Mutp53 proteins were also reported to promote nucleotide synthesis through its interaction with ETS2 to activate numerous nucleotide metabolism genes (*RRM2*, *dCK*, *TK1*, *GMPS*, *IMPDH1*, *PAICS*) involved in both the *de novo* and the salvage pathways required for nucleotide synthesis, leading to elevated nucleotide pools and the subsequent enhancement of GTP dependent protein (GTPase) activity (30, 31). Collectively, these findings highlighted the metabolic reprogramming roles of mutp53 in cancer cells.

Promoting Metastasis

Metastasis is another "hallmark of cancer" and contributes to over 90% of cancer-associated deaths (15, 32). Epithelial-to-mesenchymal transition (EMT) is the first and the most essential step of metastasis that allows the cells to change their morphology to gain enhanced migration and invasion capacity. Mutp53 were reported to promote the expression of several key EMT-related transcription factors including ZEB1, SLUG, and TWIST1 through transcriptional, post-translational and epigenetic modifications, possibly in a cell type dependent manner (33–35). In endometrial cancer tissues, mutp53 represses the expression of *miR-130b*, which negatively regulates *ZEB1* (33). In non-small-cell lung cancers (NSCLCs), mutp53 inactivates MDM2 mediated SLUG degradation and result in high SLUG and low E-cadherin expression (34). While in prostate cells, mutp53 induces the reduction of H3K27me3 repression mark on TWIST1 promoter (35). Besides, several lines of evidence suggest that p63 is an effector of mutant p53 mediated metastasis. Mutp53 forms a ternary complex with p63 and phosphorylated Smad2 in the presence of TGF-β signaling, which repressed the activation of p63 downstream metastasis suppressor genes *Cyclin G2* and *Sharp-1* (36). Mutp53 also inhibits p63 mediated inactivation of Rab-coupling protein (RCP), resulting in enhanced α5β1-integrin and EGFR trafficking to the plasma membrane and the constitutive activation of EGFR/integrin signaling and its downstream pro-metastatic Akt signaling (37, 38). Besides, the activation of RCP by mutp53 also enhances HSP90α secretion, which increases cell motility through interaction with extracellular matrix (ECM) (39). Metabolism reprogramming is also involved in mutp53 induced metastasis. Using a $p53^{R172H/+}$ mice model (R175H in human), Xiong. et al. found that the interaction between mutp53 and ETS2 also induces *Pla2g16* expression, which encodes a phospholipase that catalyzes phosphatidic acid into lysophosphatidic acid and free fatty acid, and both of which have been implicated in promoting migration and metastasis (40–42). Besides, the common polymorphism Pro72Arg at mutp53 enhances migration and metastasis of tumors through its ability to bind and regulate PGC-1α target genes, which is a key regulator in mitochondrial biogenesis and oxidative phosphorylation (43, 44).

Inducing Chemo- and Radio-Resistance

Chemotherapy and radiotherapy are currently the most widely used therapies for metastatic cancers. However, tumor cells always develop ways to evolve radio- and chemo-resistance capacity to survive these therapies, and mutation in the p53 gene is one of the crucial attempts (45). In this context, mutp53 proteins regulate the expression of several chemo- and radio-resistant genes. MDR1 (multi-drug resistance 1) encodes an energy-dependent efflux pump that mediates the resistance of tumor cells to various hydrophobic cytotoxic drugs (46). Mutp53 proteins strongly upregulate MDR1 expression through ETS1 mediated promoter binding, while the restoration of WT p53 could abolish MDR1 activity by reducing its phosphorylation (47–49). Mutp53 activates the expression of NRF2, which is known to confer both chemo- and radio-resistance (50), including chemo-resistance of cisplatin, apigenin, and radio-resistance of tumor cells (51–53). In triple-negative breast cancer cells, the cooperation between mutp53 and NRF2 was reported to activate proteasome gene transcription, resulting in resistance to proteasome inhibitor carfilzomib (54). Therefore, targeting NRF2 pathway has the potential to increase the curcumin compound induced cell death of mutp53-carrying cancer cells (55). In addition, in cells with WT p53, DNA damage caused by radiotherapy and most chemotherapeutic agents would lead to p53 accumulation and apoptosis. Whereas certain mutp53 has been reported to inhibit caspase-9 and p63/73-dependent induction of *Bax* and *Noxa*, contributing to the anti-apoptotic effects of mutp53 and the insensitivity of mutp53 harboring cells to radio- and chemo-therapies (56–58).

Emerging evidence suggests that the radio- and chemo-resistance capacity are primarily achieved by cancer stem cells (CSC) (59–61). Mutp53 proteins play vital roles in CSC formation and maintenance (62). High prevalence of p53 mutations is reported in poorly differentiated carcinomas and contributes to a stem cell-like transcriptome (63, 64). WT p53 has been reported to repress the expression of several CSC markers including CD44, c-KIT, NANOG and OCT4, while mutations in p53 would lead to loss of repression on these CSC markers, subsequent CSC transformation and the resultant enhanced radio- and chemo-resistance (65).

Facilitating a Pro-Oncogenic Tumor Microenvironment

It is now accepted that tumor progression and response to therapeutic treatments are not simply dependent on cell autonomous characteristics. The tumor microenvironment consisting mainly of ECM, stromal cells, immune cells, and blood vessels plays a key role in the tumorigenesis and chemoresistance capacity (15). Mutp53 can modulate tumor microenvironment by inducing the secretion of pro-inflammatory cytokines and angiogenesis (66, 67). The p53R248W and D281G mutants can activate the activity of matrix metalloproteinases (MMPs) by repressing the transcription of TIMP3 (68). Consistently, colorectal carcinomas expressing $p53^{R273H,V216M}$ show significant upregulation of MMP9 expression (69). The increased MMP activity results in the degradation of ECM surrounding the tumor

cells, leading to enhanced metastasis and invasiveness (68, 70). Mutp53 has also been reported to form complex with HIF1 to upregulate ECM components Viia1 collagen and laminin-γ2 to promote tumor progression (71). In addition, the crosstalk between Mutp53 and the master inflammatory regulator NF-κB pathway has been largely implicated in modulating tumor development and migration (69, 72–74), through the upregulation of a cancer-related gene signature including CXC-chemokines, interleukins (ILs) and ECM-related genes (73–75). Finally, mutp53 was reported to positively regulate the expression of pro-angiogenic factors including IL-8, GRO-α, and VEGF to promote tumor neo-angiogenesis, which is another "hallmark of cancer" (15, 76, 77).

Therapeutic Strategies for Cancer Harboring p53 Mutations

The reliance of tumors on mutp53 makes it an ideal target for cancer therapy. Therapeutic strategies targeting mutp53 can be divided into three categories, restoring the WT conformation and transcriptional activity of mutp53, targeting mutp53 for degradation, and inducing synthetic lethality (78, 79). To achieve these therapeutic goals, small molecular compounds, synthetic small peptides, CRISPR/Cas9 mediated genome editing, small interference RNAs (RNAi) as well as immunotherapies have been explored (**Figure 1**).

Small-Molecule-Compounds-Based Therapy Targeting Mutp53

Pharmaceutical targeting of mutp53 is more challenging than targeting oncogenic kinases, which can be easily inactivated by small molecule inhibitors (80). While intensively pursued, no mutp53 targeting regiment has yet been clinically approved. Several promising small molecule drugs under clinical trials are reviewed below (ClinicalTrials.gov). Some other drugs that have achieved promising results in preclinical studies have been extensively reviewed elsewhere (79, 80).

APR-246

APR-246 is a methylated analogue of PRIMA-1, which was identified as a low-molecular-weight compound that restores wildtype function of mutp53 (81, 82). While methylene quinuclidinone (MQ) is the common bioactive decomposition product of both APR-246 and PRIMA-1, the bioactivity of APR-246 is much higher, possibly due to its higher lipophilicity and cell permeability (80, 82). The covalent binding of MQ to p53 core domain, primarily *via* cysteines 124 and 277, enhances the thermostability of mutp53 and contributes to the refolding of mutp53 to WT conformation, thus enables the re-induction of p53 target genes such as *CDKN1A* (83–85). Numerous preclinical studies using rodent models have revealed the tumor suppressive effect of APR-246 on mutp53-expressing tumor cells of various origins (86–90). Furthermore, the phase I study has verified that APR-246 is safe at predicted therapeutic plasma levels with a favorable pharmacokinetic profile, and most importantly, can induce considerable p53-dependent biological effects in cancer patients with p53 mutations (91, 92). Therefore, APR-246 is considered as a promising first-in-class mutp53-targeting drug. Now, several phase II clinical trials of APR-246 are ongoing, including a systemic carboplatin combination chemotherapy with APR-246 in patients with platinum sensitive recurrent high grade serous ovarian cancer with

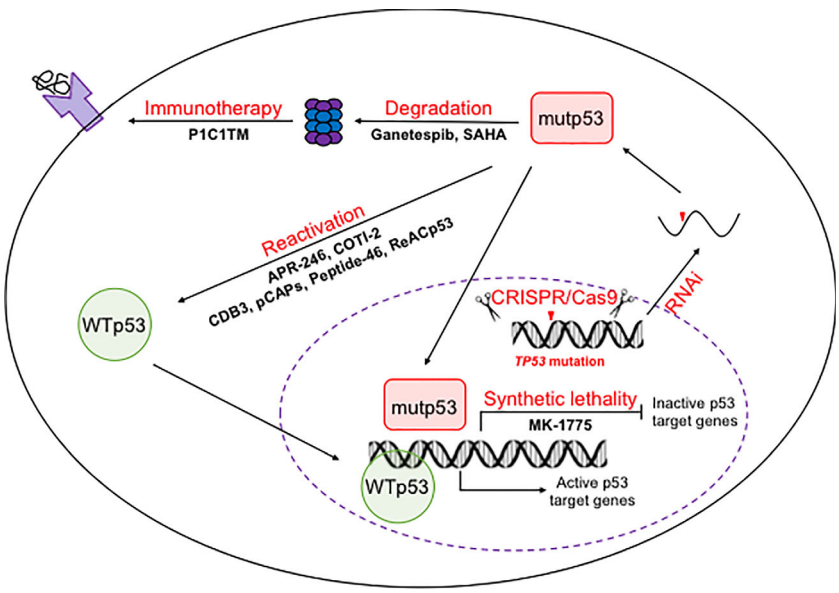

FIGURE 1 | Therapeutic strategies to target p53 mutants. On the DNA level, mutations in *TP53* allele could be reversed back to wild-type ones using CRISPR/Cas9 mediated genome editing. One the mRNA levels, mutp53 mRNA could be silenced by RNAi. On the protein level, mutp53 could be reactivated or trageted for degradation by both small molecule compounds and small peptides. The inability of mutp53 to activate its downstream target genes provides an opportunity for synthetic lethality based therapy. The mutant peptides produced by degradation of mutp53 makes immunotherapies possible.

mutated p53 (NCT02098343), a combination of APR-246 with azacytidine in p53 mutant myeloid neoplasms (NCT03072043) and a combination of APR-246 with 5-FU and cisplatin in oesophageal cancer (NCT02999893).

COTI-2

COTI-2 is a novel thiosemicarbazone derivative that is active against multiple human cancers from different origins (93). The anti-tumor activity of COTI-2 is at least partially achieved by promoting the refolding and therefore the DNA binding capacity of mutp53, leading to the reactivation of wildtype p53 target genes including *CDKN1A*, *PUMA*, and *NOXA*. Besides, MAPK and mTOR pathways are also involved in COTI-2 induced apoptosis or senescence (80, 94, 95). COTI-2 is effective at nanomolar concentrations *in vitro*, and is proved to be safe and well-tolerated in xenograft mouse models (93). Following studies revealed that COTI-2 was synergistic in combinations with cytotoxic chemotherapeutics without exerting significant toxicities *in vivo*. In addition, tumor cells resistant to chemotherapeutic agents exhibit no or little cross-resistance to COTI-2, highlighted the potential of COTI-2 in salvage treatment after current first- and second-line treatment failures (96). Based on these observations, a phase I trial of COTI-2 as monotherapy or combination therapy in gynecological tumors and head and neck squamous cell carcinoma (HNSCC) with confirmed p53 mutations is currently being performed (NCT02433626).

Ganetespib

In contrast to the relative low levels of wildtype p53 in unstressed physiological conditions, mutp53 is in most cases highly expressed in tumor cell, which is achieved by its cooperation with HSP90 chaperone machinery that inhibit the activity of its primary E3 ubiquitin ligase MDM2 and CHIP (97, 98). This hyperstabilization of mutp53 largely contributes to its dominant-negative and oncogenic GOF activities, and is the foundation of anti-tumor therapies aimed to induce mutp53 degradation. Ganetespib is a highly efficient HSP90 inhibitor (99), which is 50-fold more potent than the first-generation HSP90 inhibitor 17AAG in degrading mutp53 and killing mutp53 cancer cells (100). *In vivo* studies suggested that ganetespib extended the survival of tumor-bearing R172H (corresponding to R175H in human) and R248Q *Trp53* knock-in mice, while have no effect on their corresponding $Trp53^{-/-}$ littermates (100). Meanwhile, in $Trp53^{R248Q/-}$ mice bearing T-lymphomas, ganetespib synergizes with cyclophosphamide to suppress tumor growth and extend survival (101). However, it is worth noticing that mutp53 is not the only target of HSP90, instead, HSP90 regulates the activation and stability of a diverse array of oncogenic proteins including HER2, mutant EGFR, and mutant BRAF (99). Even though phase II clinical trials in metastatic breast cancer, malignant peripheral nerve sheath tumors and advanced non-small cell lung cancer all reported that the effect of ganetespib alone or in combination with other anti-tumor drugs did not meet the criteria for overall response rate, subgroups of these patients showed positive responses, which might be attributed to their specific genetic background (102–104). Therefore, more extensive clinical trials with ganetespib are needed.

SAHA

Histone deacetylase (HDAC) inhibitors are another group of compounds that are widely reported to reduce the levels of mutp53. SAHA (suberoylanilide hydroxamic acid) is a FDA-approved HDAC inhibitor for the treatment of T cell lymphomas (105). Recent studies found that SAHA exhibits preferential cytotoxicity for mutp53, rather than WT and null p53 cancer cells in certain kinds of human cancers, and also strongly sensitizes mutp53 harboring cancer cells to chemotherapies (100, 106, 107). Mechanistically, SAHA could destabilize mutp53 through inhibition of the HDAC6-HSP90 chaperone axis, and at the same time, inhibit the transcription of mutp53 through HDAC8 (106–109).

MK-1775

p53 is mainly responsible for the G_1/S cell cycle arrest, while in mutp53 harboring cancer cells, the abrogation of this checkpoint results in direct S phase entry even in the presence of DNA damage, making the cells more dependent on G_2/M checkpoint to maintain genomic stability (110). In this context, further inactivation of G_2/S checkpoint will lead to unscheduled mitotic entry of cells with extensive DNA damage, resulting in mitotic catastrophe (111, 112). This synthetic lethality provides an ideal opportunity for therapeutic targeting of mutp53 harboring cancer cells. Wee-1 is a tyrosine kinase that involved in DNA damage induced G_2/M cell cycle arrest by inhibiting CDK1 activity (113). Its specific inhibitor MK-1775, therefore, was reported to show amplified anti-tumor activity specifically in p53 mutant cancer cells. MK-1775 significantly elevated the efficacy of cisplatin, vorinostat (HADC inhibitor), or alisertib (aurora kinase A inhibitor) in HNSCC cells expressing high-risk mutp53 both *in vitro* and *in vivo*, while tumor cells bearing wildtype p53 displayed minimal response to MK-1775 (114–117). Consistently, MK-1775 was also reported to sensitize p53 mutant colon cancer cells to the DNA damage associated drug irinotecan (118). Currently, a randomized phase II study evaluating MK-1775 in combination with paclitaxel and carboplatin in adult patients with platinum sensitive p53 mutant ovarian cancer is ongoing (NCT01357161).

Genetic Approach to Target Mutp53

CRISPR/Cas9 and RNAi

CRISPR/Cas9-based genome editing appears to be a straightforward therapeutic strategies for tumor cells expressing p53 mutants. By directly replacing the TP53 414delC frameshift mutation locus with a functional copy, Batir et al. successfully restored the wild-type *TP53* genotype and phenotype in prostate cancer cells (119). CRISPR/Cas9 has also also employed in a p53 genetic sensor system which specifically and efficiently killed p53-deficient cancer cells (120). However, the high risk of genome instabiliy induced by CRISPR/Cas9 should be rigorously considered (121, 122). Small interference RNAs could specifically eliminate mutant p53 mRNA without affecting the wild-type one, However, the specificity and *in vivo* efficacy of such RNAi remains to be elucidated.

Small Peptides

The goal of small peptide based therapies is to restore wild-type p53 function, either by restabilization of mutp53 or inhibition of

the aggregation of mutp53. The denaturation of mutp53 at physiological temperature largely contributes to the inabilily of mutp53 to activate downstream tumor suppressive genes. Therefore, several mutp53 reactivating peptides, such as CDB3, peptide-46 and pCAPs, have been identified to restore wildtype p53 activities to cancer cells (123, 124). On the other hand, a large portion of p53 mutants have been reported to form protein aggregates, which contributes to the GOF properties that promote tumor growth. In this context, ReACp53, a cell-penetrating peptide inhibitor of mutp53's aggregation, which resembles the transactivation inhibitory domain of p63, showed promising anti-cancer effect in both ovarain and prostate cancer models *in vivo* (125–127).

Immunotherapy

While the accumulated mutp53 escapes from MDM2-mediated degradation, it can still be degraded in a MDM2-independent and proteasome-dependent pathway, generating peptides that are eventually presentated on tumor cell surface by class I molecules of the major histocompatibility comples (MHC). Therefore, mutp53 and the p53-derived mutant peptide-MHCs could serve as potental therapeutic targets for immunotherapies (128, 129). Even though peptides containing mutp53 sequences are rare due to MHC-binding restrictions, an engineered T cell receptor-like (TCRL) antibody P1C1TM, which is specific for a wild-type $p53_{125\text{-}134}$ peptide presented by the HLA-A24:02 (HLA-24) MHC allele, was reported to be able to discriminate between mutant and wild-type p53-expressing HLA-$A24^+$ cells based on antigen expression levels. This elegant interaction between intracellular mutp53 and targetable cell surface peptide-MHC complex enables efficient antibody dependent cellular cytotoxicity of mutp53 expressing cells both *in vitro* and *in vivo* (129). In the future, it is worthwhile to identify new cell surface peptides specifically derived from mutp53.

CONCLUSION

The addiction of cancer cells to mutp53 makes it an attractive target for cancer therapy. By elucidating the mechanisms of GOFs of mutp53, numerous strategies have been explored to specifically target mutp53. One highly pursued strategy is to develop small molecule compound and small peptide to restore the conformation and transcriptional activity of wild-type p53 to the mutp53. This strategy is challenging due to the relative undruggable nature of mutp53 with various thermostability or conformational structures. Therefore, high-resolution structural and functional analysis of the full length WT and mutp53 will be required to design more effective small molecule compounds and small peptides to target mutp53. However, it is noteworthy that our group recently found that hepatocellular carcinomas (HCCs) often retain the wild-type p53 to suppress oxidative phosphorylation and increase glycolysis, thereby promoting HCC progression (130). In this context, strategies aiming to restore WT p53 activities of mutp53 might instead promote tumorigenesis under certain circumstances, therefore requires rigorous validation before clinical trials. Synthetic lethality, gene editing, siRNA silencing, and immunotherapy are promising strategies to target mutp53 to treat mutp53-expressing tumors, however, these approaches all have intrinsic problems that must be optimized before clinical applications. In this context, future effort should be devoted to improve the specificity, efficacy, and safety of these promising strategies to target mutp53-expressing human cancers.

AUTHOR CONTRIBUTIONS

GZ, CP, JB, BL, CL, YX, and XF drafted the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Cancer Stemness: p53 at the Wheel

Dishari Ghatak[1†], *Damayanti Das Ghosh*[2†] *and Susanta Roychoudhury*[2*]

[1] Cancer Biology and Inflammatory Disorder Division, CSIR-Indian Institute of Chemical Biology, Kolkata, India, [2] Division of Research, Saroj Gupta Cancer Centre and Research Institute, Kolkata, India

The tumor suppressor p53 maintains an equilibrium between self-renewal and differentiation to sustain a limited repertoire of stem cells for proper development and maintenance of tissue homeostasis. Inactivation of p53 disrupts this balance and promotes pluripotency and somatic cell reprogramming. A few reports in recent years have indicated that prevalent *TP53* oncogenic *gain-of-function* (GOF) mutations further boosts the stemness properties of cancer cells. In this review, we discuss the role of wild type p53 in regulating pluripotency of normal stem cells and various mechanisms that control the balance between self-renewal and differentiation in embryonic and adult stem cells. We also highlight how inactivating and GOF mutations in p53 stimulate stemness in cancer cells. Further, we have explored the various mechanisms of mutant p53-driven cancer stemness, particularly emphasizing on the non-coding RNA mediated epigenetic regulation. We have also analyzed the association of cancer stemness with other crucial *gain-of-function* properties of mutant p53 such as epithelial to mesenchymal transition phenotypes and chemoresistance to understand how activation of one affects the other. Given the critical role of cancer stem-like cells in tumor maintenance, cancer progression, and therapy resistance of mutant p53 tumors, targeting them might improve therapeutic efficacy in human cancers with *TP53* mutations.

Keywords: GOF mutant p53, cancer stemness, differentiation, epithelial to mesenchymal transition, chemoresistance, miRNAs, therapeutic targeting

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***Correspondence:**
Susanta Roychoudhury
susantarc@gmail.com

[†]*These authors have contributed equally to this work*

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INTRODUCTION

The tumor suppressor p53 has been described as the "guardian of the genome" for its pivotal role in protecting the cells from neoplastic transformation. Apart from its classical function in cell-cycle arrest, DNA-repair, apoptosis, and senescence, it also supervises processes such as cellular plasticity, self-renewal, and differentiation (1, 2). *TP53* maintains homeostasis between self-renewal and differentiation depending on the cellular and developmental state and prevents the dedifferentiation and reprogramming of somatic cells to stem cells (2). *TP53* is frequently altered in human tumors. The majority of alterations are somatic missense mutations that occur in the DNA binding domain between amino acids 125 to 300 (3). The DNA-binding domain mutants are categorized into "contact" (R248, R273) mutants, where amino acid residues involved in making direct contact with the DNA and "conformational" mutants (R175H, G245, R249, and R282) that disrupt the p53 protein structure at a local or global scale (4, 5). These mutants not only lose the canonical tumor-suppressive functions of their wild-type counterpart but also empower cancer cells by imparting gain-of-function (GOF) properties that favor cancer cell survival and promote tumor progression (6–9).

The GOF mutant p53 proteins regulate several cellular genes and non-coding RNAs primarily as a transcription factor and confer oncogenic properties such as sustained proliferation, increased chemoresistance, invasion and metastasis, angiogenesis, deregulated cellular metabolism, genomic instability, resistance to cell death, evading immune destruction, and replicative immortality (10). In recent years, a novel function of mutant p53 in promoting dedifferentiation of somatic cells to cancer stem cells (CSCs) has gathered considerable attention. The notion that GOF mutant p53 play a major role in CSC formation was derived from the undifferentiated and chemoresistant nature of the mutant p53 tumors (11). This was further supported by the common gene signature and similar transcription factor shared among embryonic stem cells (ESCs) and undifferentiated tumors of breast and brain (12). The poor prognosis of cancer patients with p53 mutations also strengthened this belief (13). However, a few direct evidence supporting the role of mutant p53 in driving CSC phenotype came along only in the recent years (14, 15). In this review we discuss various mechanisms driving alteration of cellular plasticity upon p53 mutation and efforts to delineate novel ways to specifically target the aggressive CSCs residing in mutant p53 tumors or to obstruct mutant p53 driven conversion of somatic cells to CSCs.

STEM CELLS AND CANCER STEM CELLS

Stem cells are a rare population of cells that can perpetuate themselves through self-renewal and can give rise to mature cells of a tissue by differentiation (16). While embryonic stem cells (ESCs) are pluripotent and have the ability to differentiate into three embryonic lineages, ectoderm, mesoderm, and endoderm, adult stem cells (ASCs) being multipotent in nature can differentiate into cells of a particular lineage. For example, hematopoietic stem cells (HSCs) can generate cells of the hematolymphoid system only (16). Stem cells in tissues reside in a specific location and are responsible for homeostasis and maintenance of tissue integrity and repair of damaged tissue.

Cancer stem cells (CSCs) are a subset of tumor cells that can self-renew and differentiate to generate the heterogenous cell population in a tumor (16). CSCs and normal stem cells share the ability of persistent proliferation that maintain the CSC/stem cell pool and also generate differentiated cells that form the bulk of tumor/tissue. The heterogeneity in solid tumors has been explained by two main models. The "stochastic" or "clonal evolution" model suggests that every cancer cell present in a tumor possess the same potential to proliferate and generate a new tumor (17). On the contrary, the "hierarchical" model postulates a hierarchical organization of cells in a tumor, with a subpopulation of cells accountable for maintenance of heterogeneity in primary tumor and generating new tumors similar to the original one (16, 18, 19). This population of tumor initiating cells has been termed as cancer stem cells for their "stem-like" ability of self-renewal and differentiation.

Although, the "hierarchical" model has been widely adopted but some evidences suggest that this template is not applicable for all adult stem cell/cancer stem cell prototypes. The hierarchical model suggests that stem cells/CSCs are rare and quiescent, however, the adult stem cells residing in epidermis or intestinal crypts are abundant in their niches and can actively divide throughout their lives (20). According to the "hierarchical model" stem cells/CSCs undergo asymmetric division to form one stem cell and one daughter cell (21). However, some adult stem cells can divide to generate zero, one, or two new stem cells which compete to occupy the niche by a process called neural competition (22, 23). Moreover, these adult stem cell hierarchies are extremely plastic, implying that the daughter cells and fully differentiated cells can revert to form stem cells and occupy the niche. For example, the differentiated hepatocytes can re-enter the cell cycle and can replace lost tissue upon hemi-hepatectomy (24).

CSCs and non-CSCs undergo transitions between stem and differentiated state upon exposure to therapeutic insults or certain stimuli within the microenvironment (25–27). For example, upon radiation treatment CSCs are enriched *in vivo* which suggests that radiation induces phenotypic transition of non-CSCs to CSCs. Similarly, cisplatin treatment triggers ovarian cancer non-CSCs to acquire self-renewal property (27). Furthermore, differentiated colorectal cancer cells were found to give rise to CSCs upon NF-kB activation, APC depletion, and upon chemically induced inflammation (28, 29). The dynamic nature of the CSCs and non-CSCs were further exemplified by the study in which cell population isolated based on stem cell, basal or luminal like phenotype from a breast cancer cell line could undergo phenotypic transitions *in vitro* and generate cells of the other two types (30). Interestingly, all the subcultures grown from all the three subpopulations converged over time to the same proportion of cell types of the original breast cancer cell line indicating that the inter-conversions were stochastic and independent of the phenotype of the cell of origin. However, the phenotypes were functionally significant as only the stem-like cells formed tumors upon xenotransplantation. Cell ablation experiments have recently been used to investigate CSC plasticity in human cancer xenografts (31, 32). Using CRISPR-Cas9 approach, inducible caspase 9 (iCasp9) was inserted in the *LGR5* locus of human colorectal cancer organoids, which is a common CSC marker for colorectal cancer (31). The induction of apoptosis in xenografts produced by these organoids resulted in shrinkage of tumor. However, upon removal of the inducer, the mitotically arrested, differentiated tumor cells restored the Lgr5+ CSC population and proliferated to regenerate the tumor. This further establishes the plasticity of CSC and non-CSC population in tumors. However, in certain cancer types the hierarchical organization is proposed to be unidirectional and largely irreversible. The ablation of CSC pool in glioblastoma xenograft halted tumor growth without apparent regeneration of the CSC pool from the other non-CSC glioblastoma cells (33). Although CSCs share the core traits of self-renewal and differentiation with normal stem cells, the phenotypes of the CSCs are more complex, varying from one tumor to another and are influenced by the abnormalities occurring during neoplastic transformation.

WILD TYPE p53 CONTROLS CELLULAR PLASTICITY

Apart from the acclaimed role of p53 as the "guardian of the genome" in somatic differentiated cells, a profound function of it

has also been established in stem cells. Recent studies combined with the basic information obtained in last 25 years provide an understanding of how wild-type p53 regulate the quantity and quality of stem cells to ensure normal development and a cancer-free life. In this section we address the role of p53 in regulating embryonic stem cell and adult stem cell self-renewal and differentiation, in preventing CSC formation and in generation of induced pluripotent stem cells.

p53 Controls the Balance Between Self-Renewal and Differentiation in Embryonic Stem Cells

The tumor suppressor p53 plays a significant role in ensuring genomic integrity of embryonic stem cells and controls their proliferation, differentiation, and apoptosis. In human embryonic stem cells (hESC), p53 is present in low levels due to the negative regulation by E3 ubiquitin ligases HDM2 and TRIM24 (**Figure 1**). Acetylation of p53 at K373 by CBP/p300 leads to dissociation of HDM2 and TRIM24 and subsequent activation of p53 which in turn transcriptionally activates p21, miR-34a, and miR-145 (**Figure 1**). Induction of p21 elongates G1 phase facilitating differentiation while, miR-34a and miR-145 counteracts pluripotency by targeting Lin28a, Oct4, Klf4, and Sox2 (**Figure 1**) (34). Similarly, p53 activation by nutlin leads to transcriptional activation of p21 that cause cell cycle arrest and induces differentiation in human ESCs (35). As activation of p53 leads to differentiation of ESCs, p53 is maintained in an inactive state during self-renewal of human ESCs by Oct4 induced Sirt1 mediated deacetylation (**Figure 1**) (36).

Unlike human ESCs, mouse ESCs display high levels of p53 protein localized in the cytoplasm, which declines during organogenesis and is barely detected in terminally differentiated tissues (**Figure 1**) (37). When mESCs are exposed to reactive oxygen species (ROS), Sirt1 facilitates translocation of p53 to the mitochondria instead of nucleus and induces mitochondrial-dependent apoptosis. This blocks p53 mediated suppression of Nanog transcription and maintains ESC pluripotency (**Figure 1**) (38). Lee et al. showed that in mouse embryonic stem cells (mESCs), Aurka-mediated phosphorylation of p53 suppress p53 activity and mediates mESC pluripotency (**Figure 1**). However, when Aurka levels are low, p53 transcriptionally activates ectodermal and mesodermal genes leading to differentiation (**Figure 1**) (39). Sabapathy et al. found that undifferentiated embryonic stem cells derived from murine embryonic stem cell lines express high levels of p53 in wild type conformation. *In vitro* differentiation of these cells resulted in decrease of p53 protein and triggered a shift in its conformation to mutant form (40).

DNA damage in embryonic stem cells leads to p53 activation and subsequent differentiation (41). In hESCs DNA lesions trigger p53-dependent apoptosis and differentiation (42). Although the role of p53 in DNA damage repair in ESCs is debatable, p53 deletion has been found to increase ESC survival upon DNA damage (43, 44). DNA damage in mESCs leads to activation of p53 by phosphorylation at Ser 315 residue, which then binds to the promoter of ESC self-renewal gene Nanog and suppresses its transcription (**Figure 1**) (45). This induces differentiation of mESCs and maintains their genomic stability. Apart from DNA damage, oncogenic stress signals and stimuli such as retinoic acid also induce differentiation of mESCs (46). Interestingly, p53 has also been found to induce anti-differentiation programs in mouse ESCs in response to UV radiation mediated DNA damage by directly regulating the Wnt pathway (**Figure 1**) (47). This suggests that p53 is a crucial regulator of both pro-differentiation and anti-differentiation programs and maintains homeostasis between self-renewal and differentiation depending on the developmental state (47). The role of p53 as a pluripotency switch was elaborately explored by Ungewitter et al. (48). They found that partial expression of p53 isoform Δ40p53 led to loss of pluripotency in mouse ESCs and triggers differentiation in somatic cells. However, increased expression of Δ40p63 isoform helped in stem cell maintenance mediated by Nanog and IGF-1 receptor and other p53 family members, p63 and p73 (41). Although p53 knockout mice grow normally, they develop tumors in their adult life which suggests that p53 is involved in assuring the genetic fidelity in embryonic stage (49). The critical role of p53 in embryonic development is further supported by the developmental defects, low fertility, and spermatogenesis defects exhibited by p53 null mice (50, 51).

p53 Acts as a Barrier to Somatic Cell Reprogramming

Somatic cells can be reprogrammed to induced pluripotent stem cells (iPSCs) by overexpression of transcription factors such as *OCT4*, *SOX2*, *KLF4*, and *c-MYC* (52, 53). These factors, also known as Yamanaka factors, are highly expressed in embryonic stem cells and regulate the developmental signaling required for ES cell pluripotency. However, the efficiency of somatic cell reprogramming is considerably low, and very few cells are reprogrammed to iPSCs (54). A recent study by Zhao et al. demonstrated that siRNA mediated knockdown of p53 in human adult fibroblasts enhance the efficiency of iPS cell generation up to 100-fold even in the absence of *c-MYC* overexpression (55). Also, reduction of p53 signaling by knocking down its target gene p21, or antagonizing apoptosis induced due to reprogramming, increases efficiency of transformation (56). Functional analysis of common set of genes expressed in mouse and human fibroblasts revealed p53-p21 pathway as the roadblock to iPS cell generation (**Figure 1**) (57). Indeed, the expression of reprogramming factors activates p53 pathway which eliminates cells with DNA damage, DNA repair deficiencies and those with shortened telomeres by the activation of DNA damage response or p53-dependent apoptosis (58). However, when p53 is abrogated, somatic cells carrying persistent DNA damage or chromosomal aberrations are efficiently reprogrammed to iPS cells. This indicate that reprogrammed cells are tolerant to different types of DNA damage and p53 act as a barrier in generation of human and mouse iPS cells from suboptimal parental cells. The pro-apoptotic protein PUMA has also been found to be an independent facilitator of p53 mediated suppression of induced pluripotent stem cell generation (**Figure 1**) (59). p53 may also impede reprogramming by inducing lincRNAp21 which associates with H3K9 methyltransferase SETDB1 and DNA methyltransferase

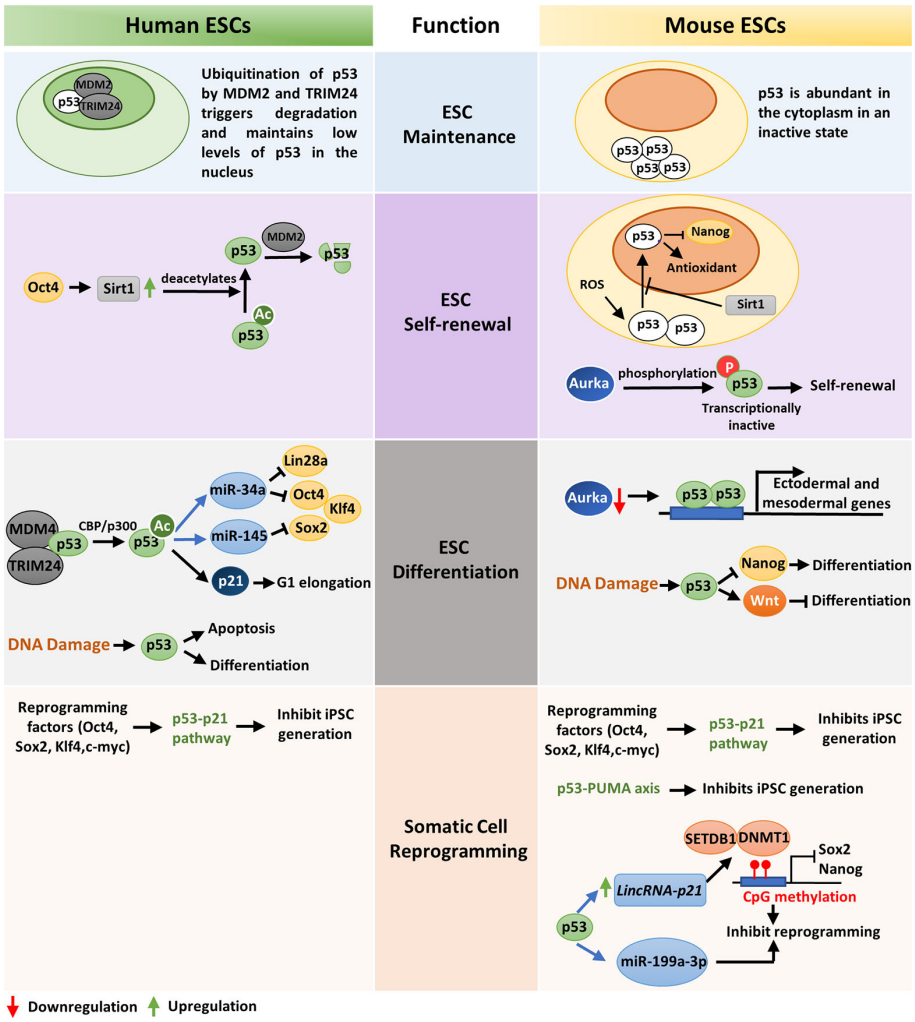

FIGURE 1 | A comparative view of wild-type p53 function in ESC maintenance, differentiation, and somatic-cell reprogramming of human and mouse: *ESC maintenance*: p53 is maintained in an inactive state in both human and mouse ESCs. In hESCs, deacetylated inactive p53 is present in low levels in the nucleus while in mESCs the inactive p53 protein is abundantly present in the cytoplasm. *ESC self-renewal*: To ensure ESC self-renewal, p53 is either prevented from entering the nucleus or maintained in an inactive state. In hESCs, Oct4 increases Sirt1 expression which in turn deacetylates p53 and promote its degradation by MDM2. This maintains a low level of p53 in the cell which is crucial to maintain stemness. Endogenous ROS induced p53 nuclear translocation in mESCs is blocked by Sirt1. This prevents p53 mediated suppression of Nanog and stem-cell phenotype is maintained. Phosphorylation and subsequent inactivation of p53 by Aurka also promotes pluripotency of mESCs. *ESC differentiation*: In hESCs, CBP/p300 mediated acetylation of p53 leads to its activation and subsequent transcription of p21, miR-34a and miR-145 which facilitates differentiation. DNA damage in hESCs also leads to differentiation or apoptosis. When Aurka levels are low in mESCs, p53 transcribes ectodermal and mesodermal genes leading to differentiation. Also, upon DNA damage, p53 primarily promotes differentiation by suppression of Nanog. However, occasionally p53 may also induce anti-differentiation pathway by activating Wnt. *Somatic-cell reprogramming*: Reprogramming of somatic cells to induced pluripotent stem cells (iPSCs) is primarily inhibited by the p53-p21 pathway in both human and mouse. Additionally, p53 may also induce lincRNAp21 or miR-199a-3p to inhibit reprogramming. The p53-PUMA axis has also been found to suppress reprogramming of mouse embryonic fibroblasts (MEFs).

DNMT1 and maintains CpG methylation at Sox2 and Nanog promoters (60). Moreover, p53 may upregulate miR-199a-3p which in turn impose G1 arrest, to decrease reprogramming efficiency (**Figure 1**) (61). Although permanent suppression of p53 during iPS cell generation may have deleterious effects on the genomic stability of the reprogrammed cells, transient knockdown of p53 may be useful in efficiently producing integration-free iPS cells for future medical use (62).

p53 Promotes Differentiation in Adult Stem Cells

TP53 is also a critical regulator of adult stem cell differentiation. Zheng et al. reported that downregulation of Myc by the cooperative actions of p53 and PTEN is crucial for differentiation of murine neural stem cells (NSCs) (63). p53 was found to control proliferation of NSC through inhibition of Gli activity and nuclear localization, the effector of hedgehog signaling pathway

(**Figure 2**) (64). Gli in turn repress p53 by activation of Mdm2, forming a homeostatic inhibitory loop (64). The hedgehog signaling pathway can also drive self-renewal through activation of Nanog which is otherwise suppressed by p53 (**Figure 2**) (65, 66). Altogether, the Nanog-Gli-p53 axis determines NSC self-renewal and differentiation. In p53-deficient mouse astrocytes Nanog is uninhibited and promotes dedifferentiation to produce cancer stem-like cells (67). p53 deficiency also elevate the rate of neurosphere formation from the olfactory bulb cells of mouse embryo indicating that self-renewal is enhanced by loss of p53 (68). p53 also play a crucial role in regulating self-renewal and differentiation of mesenchymal stem cells (MSCs) (69). MSCs derived from p53KO mice show augmented proliferation, increased differentiation rate, and a predisposition to transformation (70). Although primary mouse bone marrow stromal cells (mBMSCs) derived from wild-type p53 or p53 knockout mice have differentiating capacity into osteogenic, adipogenic, and chondrogenic lineages, enhanced osteogenic differentiation has been found only in the absence of p53 (71). This is due to increased levels of Runx2 in p53 knockout mice, which remains suppressed by the elevated expression of miR-34 family in wild type p53 cells (71). Hence p53-deficient mBMSCs are more closely related to human osteosarcoma (71).

Wild type p53 has also been found to compromise CSC properties by directly repressing CSC markers or indirectly by inducing certain miRNAs. For example, p53 repress CD133 by directly binding to its promoter and recruiting HDAC1 (**Figure 2**). Depletion of CD133 suppresses core stemness factors Oct4, Nanog, Sox2, and c-Myc and promotes differentiation (72). Likewise, p53 suppress tumor formation by inhibiting the expression of the CSC marker CD44 by binding to a noncanonical p53-binding site on its promoter (**Figure 2**) (73). Further, induction of miR-34a by p53 functionally targets the CSC marker CD44, thereby inhibiting prostate cancer regeneration and metastasis (**Figure 2**) (74). To facilitate pluripotency, cancer stem cells keep wild type p53 levels in control. For instance, the hepatic cancer stem cell population is maintained by removal of mitochondria by autophagy. This eliminates mitochondria-associated p53 which would otherwise be activated by PINK1 to mediate suppression of Nanog (**Figure 2**) (75). Interestingly, Flesken-Nikitin et al. found that alteration of p53 status of cancer-prone SCs residing in ovarian-surface epithelium enhanced their transformation potential (76). To prevent oncogenic transformation, p53 activity is maintained by certain proteins like NUMB, a cell-fate determinant and tumor suppressor. Apart from promoting asymmetric cell division, NUMB associates with p53 and MDM2 in a tricomplex preventing ubiquitination and degradation of p53 (77). Hence, loss of NUMB in breast cancer cells leads to decreased p53 levels and increased activity of NOTCH receptor which confers increased chemoresistance (77). In a similar study, loss of p53 in mammary SCs was found to promote symmetric cell-divisions leading to increased self-renewal property and subsequently contribute to tumorigenesis (**Figure 2**) (78). Further, the human p53 isoform Δ133p53β lacking the transactivation domain was observed to promote CSC features in breast cancer cell lines by expression of Sox2, Oct3/4, and Nanog in a Δ133p53β dependent manner (79).

Further, p53 sensitizes cells to drug induced apoptosis by downregulating the multidrug resistance gene, *MDR1* (80, 81). Additionally, p53 upregulates miR-34a that represses Notch (**Figure 2**) and anti-apoptotic Bcl2 thereby promoting differentiation and apoptosis (82). Therefore, it can be concluded that wild type p53 functions to maintain a balance between self-renewal and differentiation to maintain tissue integrity, which is lost upon p53 mutation.

p53 MUTATION IMPARTS STEM-LIKE PROPERTIES TO CANCER CELLS

Any mutation of p53 (deletion and GOF missense) have the loss of wild-type function as the first consequence. The loss of tumor suppressive functions of p53 triggers multipotent/unipotent adult cells to dedifferentiate and acquire pluripotency which results in disturbances in tissue hierarchy. With the advent of reprogramming era, it was further highlighted that p53 loss promote dedifferentiation and reprogramming under favorable conditions. p53 inactivating mutations in tumors results in increased expression of CSC markers and sphere forming ability. Certain p53 missense mutants further promote these phenotypes aggravating the malignant condition.

p53 Inactivation Leads to Cancer Stemness

Although majority of tumors harbor p53 *loss-of-function* mutation (missense and truncation mutations) or functional inactivation of p53 pathway, it is more prominently correlated with dedifferentiated sarcomas and carcinomas (83). For instance in breast cancer, p53 mutation is frequently correlated with high-grade tumor types including poorly differentiated basal-like tumors (84–87). Pinho et al. revealed that pancreatic acinar cells with homozygous deletion of p53 show stemness features such as enhanced sphere formation, increased expression of CSC markers (Ptf1a,Pdx1, Cpa1, c-Myc, Sox9, and Hnf1b) and stem cell regulators like Bmi1 and Klf4 as compared to cells with wild type p53 (**Figure 2**) (88). In accordance, a later study demonstrated that p53-miR-200 axis negatively regulates Sox2, and counteracts NFATC1-Sox2 mediated dedifferentiation of pancreatic adenocarcinoma cells (89). Association of p53 inactivation and loss of differentiation characteristics has also been reported in AML and lung cancer (**Figure 2**) (90, 91). Furthermore, p53 loss was found to trigger dedifferentiation of mature hepatocytes to pluripotent cells by the activation of SC marker *Nestin*, which remains suppressed in wild-type p53 bearing cells (**Figure 2**) (92). Mammary stem cells with p53−/− and p53+/− formed larger and more number of mammospheres compared to p53+/+ cells (93). Moreover, tissue-specific adult stem cells of mouse mammary epithelium, which are not pluripotent but maintain tissue homeostasis, become tumorigenic in presence of p53 deletion (78). An interesting study by Mizuno et al. propounded that breast and

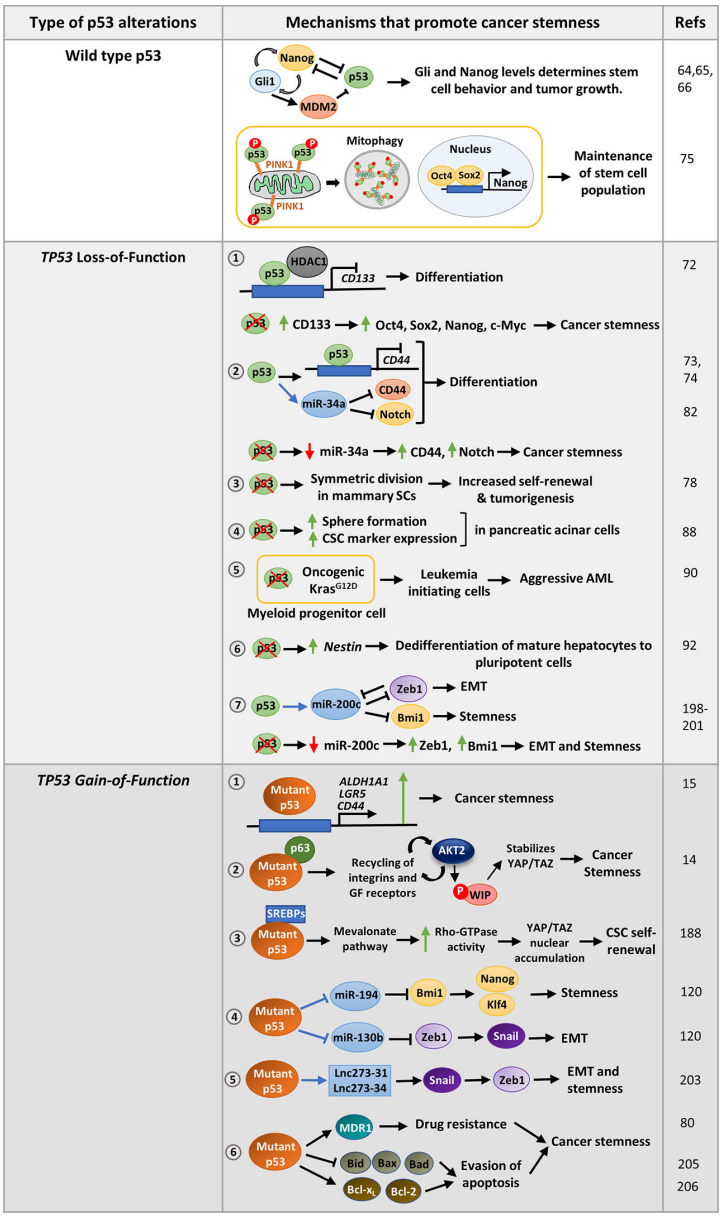

FIGURE 2 | Mechanisms that promote stemness in cancer cells harboring wild-type p53, p53 with loss-of-function mutations or gain-of-function missense mutations: Wild-type p53 modulates the Nanog -Gli positive feedback loop in neural stem cells to control pluripotency. On the contrary, Nanog suppresses p53 activity while Gli activated by Nanog inhibits p53 by activating Mdm2 to promote pluripotency. In hepatic cancer, the stem cell population is maintained by removing mitochondria-associated p53 through mitophagy. *TP53* LOF mutations promote various mechanisms that confer stemness phenotype to cancer cells. 1. p53 loss upregulates CD133 which subsequently promotes CSC marker expression and confers stemness. 2. p53 suppresses the cell-surface marker CD44 either by binding to its promoter or by upregulating miR-34a. p53 loss results in increased expression of CD44 and Notch leading to cancer stemness. 3. Loss of p53 also promotes symmetric division of mammary SCs thereby promoting tumorigenesis. 4. Homozygous deletion of p53 in pancreatic acinar cells promotes sphere formation, CSC marker expression as compared to cells with wild type p53. 5. p53 inactivation strongly cooperates with oncogenic Kras mutation in myeloid progenitor cells to induce aggressive AML. 6. p53 loss may also derepress SC marker Nestin to promote differentiation in mature hepatocytes. 7. p53 induces epithelial differentiation by activation of miR-200c. Loss of p53, leads to decreased miR-200c levels and increased expression of its target genes leading to EMT and stemness. *TP53* GOF mutations promote cancer stemness by regulating several pathways. 1. Mutant p53 can directly activate CSC markers such as ALDHA1, CD44, and LGR5 to promote stemness. 2. It may regulate Wasp-interacting protein (WIP) that regulates YAP/TAZ stability. 3. Mutant p53 can also promote self-renewal of breast cancer cells by inducing nuclear localization of YAP/TAZ by activating mevalonate pathway. 4. Mutant p53 transcriptionally represses miR-130b and miR- 194, the negative regulators of Zeb1 and Bmi1 respectively, to promote EMT and stemness 5. p53-R273H upregulates lncRNAs, lnc273-31, and lnc273-34 implicated in EMT and CSC maintenance in colorectal cancer cells. 6. GOF mutant p53 promotes typical CSC features of enhanced drug-resistance and prolonged survival by upregulating multidrug resistance gene MDR1, anti-apoptotic genes Bcl-2 and Bcl-xL, and inhibiting pro-apoptotic genes Bax, Bid, and Bad.

lung tumors with functionally compromised wild-type p53 have gene-expression pattern like ESCs (84). They also observed that breast tumors with very low ARF levels correlated with high scores for ESC signature. As ARF inhibits MDM2, low level of ARF results in high MDM2 activity and low levels of p53 which induce the SC phenotype. ARF has been found to be repressed by the polycomb complex protein Bmi1 that maintains stem cell self-renewal by maintaining low p53 protein level (94). Besides, loss of downstream effector p21 also enhances tumorigenesis in p53 deleted stem cells (95). In light of these observations one can speculate that p53 loss promote expression of a set of genes that cause reversion of the cells from terminally differentiated state to a more stem-like state that enhance tumor growth. Even when p53 is functional, deregulation of genes modulating p53 pathway can also trigger a similar phenotype.

p53 Gain-of-Function Mutation Promotes Cancer Stemness

The most frequently occurring mutations in p53 are missense point mutations that cluster in the DNA binding domain region. There are six amino acid residues, termed as "hotspots," which are commonly altered by such mutations. These mutations not only result in loss of tumor suppressive functions of p53 but also promote several oncogenic phenotypes. Hence, they are known as "*gain-of-function*" (GOF) mutations. Although the GOF mutant protein lack DNA-binding ability, they can piggyback on other transcription factors to regulate expression of a large number of genes and non-coding RNAs. In this section we will discuss the different oncogenic properties conferred by GOF mutant p53 and its role in regulating stemness of cancer cells.

Oncogenic Properties of GOF Mutant p53

The GOF mutant p53 proteins can sense the extrinsic and intrinsic stress conditions of transformed cells and synchronize adaptive responses that support tumor growth and sustenance (96). These proteins help cancer cells to cope with stress generated during tumorigenesis, such as hyperproliferation induced DNA damage, oxidative and proteotoxic stress, physical constraints, nutrient fluctuations, stromal cues, and anti-tumor immune response by promoting oncogenic *gain-of-function* phenotypes (96).

One of the distinctive stress responses of mutant p53 bearing cells is their ability to resist cell death as well as chemotherapeutic drugs insults (97, 98). This gain-of-function property of mutant p53 was revealed in 1995 when Lotem and Sachs observed that mutant p53 expression could inhibit c-Myc induced apoptosis in leukemic cells (99). Various proteins and signaling pathways are implicated in mutant p53 mediated resistance to chemotherapeutic drugs. For instance, mutant p53 driven activation of NF-kB (**Figure 3A**) or increased expression of MGMT or SLC25A1 (**Figure 3A**) confer increased resistance to etoposide, temozolomide, and cisplatin, respectively (100–102). Further, mutant p53 can interact with PELP1 to promote resistance to platinum-based drugs in triple negative breast cancer (103). A recent study by Alam et al. reveals GOF mutant p53 upregulates EFNB2 and activates ephrin B2 reverse signaling to impart enhanced chemoresistance to colorectal cancer cells (**Figure 3A**) (104). Mutant p53 also protects the cancer cells from oxidative and proteotoxic stress. For instance, it suppresses NRF2 which regulates the expression of antioxidant proteins (**Figure 3A**) (105). Mutant p53 also promotes the function of HSP1 by direct (binding) or by indirect (EGFR/ErbB2 signaling) mechanisms (**Figure 3A**) (106). As anti-apoptotic and proliferative signaling are closely linked, many molecules driving proliferation together with mutant p53 also promote chemoresistance. These include p63, p73, KLF17, REG-γ proteosome pathway and PTEN signaling pathway through Bcl-XL (**Figure 3A**) (107–110). Transcriptional de-regulation of certain miRNAs by mutant p53 may also confer chemoresistance. For instance, upregulation of miR-128-2 that targets E2F5 and downregulation of miR-223 which targets *STMN1* confers resistance to chemotherapeutic drugs (**Figure 3A**) (111, 112).

The most extensively studied function of GOF mutant p53 is however its role in promoting invasion and metastasis of cancer cells. Mutant p53 implicate various context and tissue dependent mechanisms to promote cancer cell invasion and metastasis. Mutant 53 can promote invasion and loss of directionality of migration by enhancing integrin and epidermal growth factor receptor (EGFR) trafficking which results in constitutive activation of integrin/EGFR signaling (**Figure 3A**) (113). Importantly, mutant p53 can bind to TAp63 to interfere its function leading to decreased expression of metastasis-inhibiting genes such as Sharp1, CyclinG2, and Dicer (**Figure 3A**) (114, 115). In mutant p53 bearing cells, TGF-β acts in concert with oncogenic Ras to form a complex consisting of mutant-p53-p63 and Smads (114). The formation of this complex inhibits p63 functions and expression of its target genes Sharp1 and Cyclin G2 which are essential mediators of p63-mediated antagonism towards TGFβ signaling (114). Further, mutant p53 inhibits TAp63 mediated transcriptional activation of Dicer leading to an overall depletion of miRNA processing and enhanced metastatic potential (115, 116). Mutant p53 mediated repression of p63 function can also modulate the expression of certain miRNAs involved in invasion and metastasis such as let-7i, miR-155, miR-205, miR-130b, and miR-27a (**Figure 3A**) (117–121). Various transcription factors such as NF-Y, SREBPs, ETS, and EGFR1 play crucial role in mutant p53 driven invasion and metastasis. In pancreatic cancers, mutant p53 activates the NF-Y transcription complex by releasing p73, resulting in transactivation of PDFGR-β (**Figure 3A**), promoting cell migration, while in glioblastoma PTEN promotes the association of mutant p53 with NF-Y to induce expression of Myc and Bcl-XL (110, 122). Mutant p53 in association with NF-Y and p300 can transactivate *EFNB2* to promote EMT *via* Src/Fak signaling (**Figure 3A**) (104). Binding of mutant p53 to ETS2 can promote expression of Pla2g16 or nucleotide synthesis genes required for invasion depending upon the cancer type (**Figure 3A**) (123, 124). Furthermore, the binding of mutant p53 to EGR1 promotes MYO10 expression which drives breast cancer cell invasion (**Figure 3A**) (125). Interaction of mutant p53 to SREBPs activates mevalonate pathway that promotes invasion in breast cancer cells (**Figure 3A**) (126). A recent

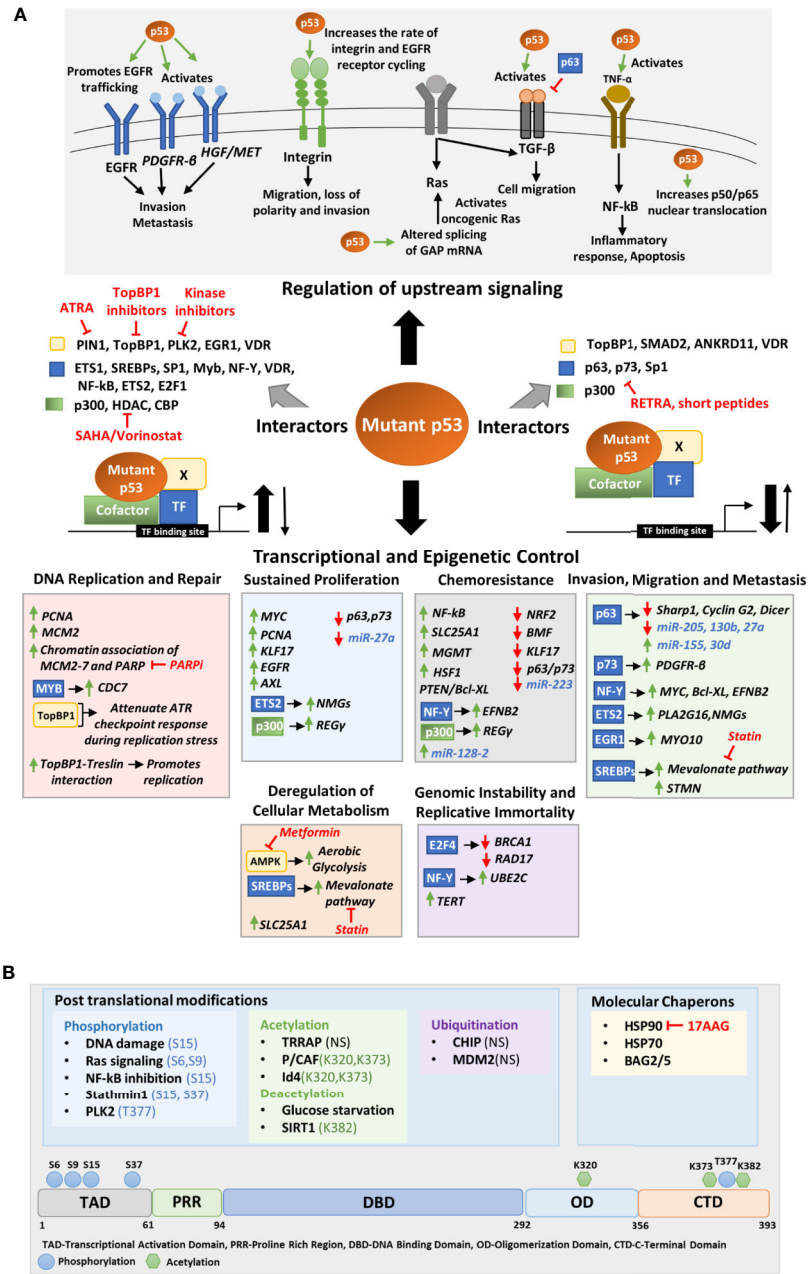

FIGURE 3 | **(A)** Molecular mechanisms of mutant p53 mediated deregulation. The upper panel depicts the upstream signaling pathways deregulated by mutant p53 to promote oncogenesis. The middle panel portrays the different transcription factors, cofactors, and other proteins to which mutant p53 may interact to either enhance or inhibit their binding to the target gene promoter. The lower panel shows the transcriptional and epigenetic targets of mutant p53 classified according to the phenotype they alter. **(B)** Upstream signals that regulate mutant p53. The upper panel shows the various post translational modifications and chaperons that regulate mutant p53 stability. The modified residues if known, have been mentioned. In others it is not-specified (NS). The lower panel shows the residues in the mutant p53 protein where post-translational modifications occur. Drugs that target interacting proteins of mutant p53, downstream pathways and upstream regulators have been indicated in red in both panels **(A, B)**.

study by Capaci et al. showed that mutant p53 can interact with HIF1α to induce miR-30d expression which promotes tubulo-vesiculation of Golgi apparatus leading to enhanced vesicular trafficking and secretion (**Figure 3A**) (127). This potentiates the deposition and remodeling of extra-cellular matrix enhancing metastatic colonization and tumorigenesis (127).

One of the important hallmarks of cancer is the process of formation of new blood vessels from existing vasculature or

angiogenesis. Mutant p53 promote tumor neo-angiogenesis through the induction of ROS and Hif1-α which induces the expression of pro-angiogenic factor VEGFA (128). Also, the upregulation of ID4 by mutant p53, promotes increased levels of pro-angiogenic cytokines such as IL-8 and Gro-α (129). The increased blood vessel formation in mutant p53 xenografts in comparison to tumors expressing wild type p53, suggests that mutant p53 plays a crucial role in promoting angiogenesis both *in vivo* and *in vitro* (128).

Cancer cells depend on glycolysis to fulfil the energy requirements for continuous growth and proliferation. Several evidence demonstrate that mutant p53 promotes glycolysis and reprograms the cellular metabolism of cancer cells. Zhou et al. showed that mutant p53 binds to novel interacting partner AMPKα in glucose starvation conditions and inhibits its activation by other kinases leading to increased aerobic glycolysis, lipid production, and cell growth (**Figure 3A**) (130). Mutant p53 also increases glucose uptake by triggering translocation of glucose transporter GLUT1 to plasma membrane (131). The increased energy required by the mutant p53 bearing cell during invasion and metastasis is provided by enhanced glycolysis through mutant p53-AMPK binding and mutant p53-SREBP binding which induce expression of mevalonate pathway enzymes (**Figure 3A**) (130, 132). Further, the transcriptional activation of mitochondrial citrate transporter SLC25A1 increases fatty acid and sterol biosynthesis and oxidative phosphorylation (**Figure 3A**) (102).

Mutant p53 promotes the expression of oncogenes such as MYC (110, 133), PCNA (134), KLF17 (108), EGFR (121, 135), and AXL (136), and simultaneously inhibits the function of tumor suppressors like the p53 family proteins, p63 and p73 (107, 137, 138) to sustain continuous proliferation of cancer cells (**Figure 3A**). The ablation of mutant p53 in mouse xenografts resulted in significant reduction of tumor growth suggesting the crucial role of mutant p53 in tumor growth *in vivo* (139). Further, mutant p53 regulation of several nucleotide metabolism genes (NMGs) such as DCK, TK1, TYMS, RRM1/2, and GMPS is required for sustained proliferation and reduced replication stress (**Figure 3A**) (124). Mutant p53 can also promote proliferation by inducing the REG-γ proteosome pathway in association with p300 (**Figure 3A**) (109).

Cancer cells utilize a higher number of replicative origins than normal cells (140). Polostkaia et al. first suggested that DNA replication might be a crucial target of mutant p53 (141). They found that mutant p53 not only upregulates two crucial replication factors, viz. PCNA and MCM2 but also stabilizes their chromatin association in breast cancer cells (**Figure 3A**) (141). A further study reported that mutant p53 enhance the association of mutant p53 and PARP on the replicating DNA (**Figure 3A**) (142). Another report by Datta et al. showed GOF mutant p53 co-operates with an oncogenic transcription factor Myb to transactivate Cdc7 in cancer cells which in turn promote Cdc7/Db4 complex formation leading to increased origin firing (**Figure 3A**) (143). GOF mutant p53 can bind to TopBP1 and attenuate ATR checkpoint response during replication stress (**Figure 3A**) (144). Moreover, it can override the Cdk2 requirement to promote replication by facilitating the interaction between TopBP1 and Treslin (**Figure 3A**) (144). GOF mutant p53 also has been found to inhibit proper restart of stalled or damaged replication forks thus driving genomic instability (145). Mutations in p53 have been associated with dysfunctional checkpoint or altered DNA repair pathways that lead to genomic alteration such as aneuploidy, chromosome translocations and amplifications (**Figure 3A**) (146–148). Mutant p53 also suppress crucial DNA repair proteins such as BRCA1 and RAD17, as a result the cell progresses with the damaged DNA leading to aneuploidy and other genomic alterations (**Figure 3A**) (149). Moreover, mutant p53 has been found to transactivate telomerase maintenance gene hTERT which might be the reason behind altered telomere length and architecture in mutant p53 bearing cells (**Figure 3A**) (146, 150).

Inflammation has been found to promote tumorigenesis by several means and has been characterized as one of the enabling hallmarks of cancer (151, 152). While wild type p53 suppresses inflammatory response by inhibiting the production of cytokines and antagonizing NF-kB activity, mutant p53 on the other hand enhances NF-kB activity in response to TNF-α and promotes inflammation (**Figure 3A**) (152–154). Further, mutant p53 together with c-MAF promote IL1-Ra expression and sustain inflammatory signaling (155). The sustained activation of NF-kB signaling by mutant p53 not only elevate inflammatory response but also protects the cancer cells from cytotoxic effects of tumor microenvironment by activating pro-survival pathways. Mutant p53 can also alter other biological processes to promote oncogenesis. A recent work demonstrated that mutant p53 alters RNA splicing by upregulating the splicing regulator hnRNPK (156). This promotes alteration in GTPase-activating protein (GAPs), the negative regulators of RAS family members, leading to heightened KRAS activity in pancreatic ductal adenocarcinoma (**Figure 3A**) (156).

The stress-responses associated with tumorigenesis represent the common hallmarks of cancer. Mutant p53 support cancer cell survival and proliferation by safeguarding them from the various oncogenic stress and was aptly called "guardian of the cancer cell" (96). These adaptive mechanisms of mutant p53 may explain addiction of cancer cells to mutant p53.

Regulation of GOF Mutant p53 by Upstream Signals

The GOF mutant p53 is regulated by various oncogenic stress signals. As mutant p53 lacks the ability to transactivate the ubiquitin ligase MDM2, it was considered that it would be accumulated in both normal and cancer tissues. However, studies with p53 knock in mice shows that its cellular levels vary from being low in normal tissues to high in cancer tissues (157). Different studies have revealed that inherently unstable mutant p53 can be stabilized by genotoxic stress (ionizing radiation, ROS), loss of tumor suppressor proteins (e.g. P16INK4A, PML) and oncogenic insults (Myc, KRas, ErbB2) (158, 159).

Mutant p53 stability and activity are primarily altered by post-translational modifications (PTMs), ubiquitin ligases and specific chaperons (**Figure 3B**). Like wild type p53, GOF mutant p53 can

also be post-transcriptionally modified by a variety of genotoxic and cellular stress signals. While these stress signals stabilize wild type p53 to suppress tumorigenesis, they stabilize mutant p53 to exacerbate tumor malignancy. DNA damaging agents such as Gemcitabine has been demonstrated to phosphorylate mutant p53 (R273H) at serine 15 which leads to nuclear accumulation of mutant p53 and increases chemoresistance (160). Chronic S15 phosphorylation of mutant p53 has been found in tumors where DNA damage signaling is constitutively activated (161, 162). Activated Ras signaling promotes phosphorylation of mutant p53(R280K) at S6 and S9, which then associate with Smad2 and TP63 to inhibit the metastasis suppressor function of the latter (114). Further, NF-kB inhibition by overexpression of IkB also results in S15 phosphorylation of mutant p53 *via* GADD45α mediated JNK1 activation (163). Additionally, stathmin1 associated with microtubule dynamics and destabilization, may phosphorylate mutant p53 at S15 and S37 and contribute to its stability (164). DNA damage induced polo-like kinase 2 (PLK2) can also phosphorylate mutant p53 (R175H, R273H) at C terminal serine residue T377, leading to enhanced binding to p300, increased acetylation and GOF activity (165). Mutant p53 acetylation also plays a role in accumulation and GOF activity of mutant p53 (166). According to a report by Minamoto et al., mutant p53 is hyperacetylated at K320, K373, and K382 in multiple cancer cell lines (167). Acetylation of K382 on mutant p53 R273H has also been reported in multiple colon cancer cell lines (168). Jethwa et al. showed that TRRAP, which recruits histone acetyltransferases to chromatin during transcription and DNA repair also stabilize different p53 mutants through inhibition of MDM2-proteasome axis in Burkitt lymphoma (169, 170). On the contrary, Id4 induced interaction of mutant p53 and p300/CBP (P/CAF) promotes acetylation at K320 and K373 resulting in increased expression of p21, BAX, and PUMA leading to apoptosis (171). This suggests that acetylation at K320 and K373 can alter the structure of mutant p53 and restore wild type p53 functions. Mutant p53 stability is also regulated by glucose levels. Glucose deprivation cause deacetylation at C terminal lysine residues and trigger mutant p53 degradation and autophagic cell death (172). Activation of SIRT1 deacetylase by YK-3-237, leads to reduced mutant p53 levels and triggers apoptotic cell death (173). Ubiquitination of mutant p53 also play a crucial role in regulating its stability and subcellular localization. While polyubiquitination of mutant p53 leads to its degradation, monoubiquitination may alter the subcellular localization of mutant p53 affecting its GOF activity (174). DNA damage induced ATM mediated phosphorylation of mutant p53 R175H at S15 results in monoubiquitination by MDM2 instead of polyubiquitination.

Molecular chaperones, such as the heat shock proteins (HSPs) are also known to bind to mutant p53 to refold, stabilize or degrade it (175–177). For example, HSP90 play a crucial role in stabilizing mutant p53. It may form a complex with mutant p53 and MDM2 to block their ubiquitination mediated degradation or may form a complex with mutant p53 to prevent aggregation of mutant p53 by inhibiting MDM2 and CHIP in multiple cancer cell lines (178, 179). Recently, Ingallina et al. showed that mechanical cues such as stiffness of the extracellular matrix trigger RhoA dependent remodeling of actin and actomyosin contractility which leads to mutant p53 accumulation by HDAC6/HSP90 axis (180). HSP70 is also involved in mutant p53 stabilization and degradation (181, 182). HSP70/HSC70 complex can recognize misfolded mutant p53 proteins and promotes its CHIP mediated ubiquitination and degradation when HSP90 activity is inhibited (181). Another member of HSP70 family, mortalin, also binds to mutant p53. Knockdown of mortalin results in nuclear translocation of mutant p53 and triggers apoptosis in HCC cell line, PLC/PRF/5 (183). However, whether mortalin inhibition restores wild type p53 function is not clear. Other than the HSPs, BCL-2 associated anthanogene (BAG) family proteins also interact with mutant p53 to promote its GOF activity by inhibiting ubiquitination mediated degradation by MDM2 and CHIP (184, 185).

Stabilization of mutant p53 promotes its gain-of-function activities. Therefore, disrupting its stability by therapeutically targeting chaperons and other proteins that impart stability to mutant p53 might be beneficial in treatment of aggressive mutant p53 tumors.

Impact of GOF Mutations on Cancer Stemness

Enhanced cancer stemness phenotype has emerged as a crucial oncogenic property of mutant p53 in recent years. The novel *gain-of-function* property of mutant p53 to enhance somatic cell reprogramming efficiency was first proposed by Sarig *et al.* in 2010 (186). They showed that GOF-mutant p53 bearing mouse embryonic fibroblasts (MEFs) reprogrammed more efficiently than p53 knockout MEFs (186). This indicates that GOF mutant p53 not only prevent elimination of sub-optimized cells by apoptosis but also facilitate in acquisition of pluripotency. Furthermore, while reprogrammed cells with p53 deficiency formed differentiated teratomas *in vivo*, those with GOF mutant p53 formed undifferentiated malignant tumors, implying that it confers oncogenic properties to the reprogrammed cells (186). A few years later, Grespi *et al.* identified a set of miRNAs whose expression altered in a p53-dependent manner during transition of mouse embryonic fibroblasts to induced pluripotent stem cells (187). The role of these miRNAs can further be investigated to determine their role in regulation of mutant p53 driven stemness. A recent study by Solomon et al. propounded that mutant p53 expressing colorectal cancer cell lines harbor an increased population of CD44, Lgr5, and ALDH positive cancer stem cells (15). Further experimental evidences showed that mutant p53 transcriptionally upregulates these CSC markers to promote cancer stem cell population in colorectal cancer cells (**Figure 2**) (15). In another study, Escoll et al. proposed that GOF mutant p53 promotes cancer stemness in glioblastoma and breast cancer cells by activating PI3K/AKT2-mediated integrin or growth factor (GF) receptor cycling. This promotes phosphorylation of WASP-interacting protein (WIP) by AKT2 which in turn stabilizes YAP/TAZ, and supports cancer stem cell survival and phenotypic maintenance (**Figure 2**) (14). Mutant p53 can also induce YAP/TAZ nuclear localization by interacting with SREBP and activating the mevalonate pathway (188). The mevalonate

cascade produces geranylgeranyl pyrophosphate which activates Rho-GTPases that in turn activate YAP/TAZ and promotes self-renewal of breast cancer cells (188). Apart from these discrete studies, the molecular mechanism of mutant p53 mediated stemness phenotype is largely unexplored. As cancer stem cell phenotype is extensively driven by epigenetic factors, especially miRNAs, it would be interesting to investigate the GOF mutant p53 altered miRNAs for their possible role in stemness (189).

The major oncogenic properties of enhanced metastasis, chemoresistance and angiogenesis conferred by GOF mutant p53 are also integral to cancer stem cells. Hence, understanding the molecular and phenotypic characteristics common to CSCs and GOF mutant p53 cells might unravel new mechanisms by which these p53 mutants promote stem-like phenotype in cancer cells.

Association With EMT

During development, embryonic cells possessing high degree of cellular plasticity undergo reversible transformations and migrate long distances to form tissues and organs. To facilitate migration, the epithelial cells acquire mesenchymal characteristics by a process known as epithelial to mesenchymal transition (EMT). Upon reaching their destination, they revert to epithelial phenotype by the process of mesenchymal to epithelial transition (MET) to settle, proliferate, and differentiate into different organs (190). These key developmental programs are often reactivated in cancer cells which lead to cancer invasion and metastasis. However, unlike in embryogenesis, EMT associated with cancer involves intravasation of delaminated cells into blood and lymphatic vessels and subsequent extravasation to colonize at distant sites. EMT is triggered by many extracellular signals and agents such as members of the transforming growth factor β (TGF-β)/bone morphogenetic protein (BMP) family, Wnt, Notch, epidermal growth factor, fibroblast growth factor, hypoxia, UV light, nicotine, and many others (191). Such signals stimulate the activation of certain transcription factors (TFs) such as Snail, Twist, Zeb, and others which may act independently or in combination to suppress epithelial phenotype and enhance mesenchymal traits such as motility, ability to degrade basement membrane and extracellular matrix (192, 193).

Metastasis involves two phases, the first involves dissemination of cancer cells from the primary site and translocation to a distant organ and second, the ability of the cancer cells to develop a tumor at the secondary site (194). At both the levels the critical role of CSCs is obvious. Primarily, the ability of the disseminated cells to seed secondary tumor and differentiate into non-stem cells are the very traits of self-renewal and tumor-initiating ability, that define CSCs. The migrating cancer cells also exhibit other features of CSCs, namely cell motility, invasiveness, and increased chemoresistance (194). Brabletz et al. termed the metastasizing cell population bearing stemness features as "migratory cancer stem cells" and proposed that they arise from stationary cancer stem cells through the gain of EMT phenotype (195). On the contrary, Chauffer et al. proposed that the presence of two CSC population in tumor; the intrinsic CSCs that are inherently present in the tumor and induced CSCs that arise from differentiated tumor cells as a consequence of EMT signaling (194). There are several reports of acquisition of stem-like features in cancer cells upon induction of EMT. Mani et al. found that induction of EMT trigger expression of stem cell markers in addition to acquisition of mesenchymal traits (196). Furthermore, the cells undergoing EMT exhibited similar mammosphere forming ability as the stem cells isolated from culture. Similarly, Morel et al. reported that EMT induction accelerate the transition of $CD44^{low}CD24^{+}$ cells to $CD44^{+}CD24^{-}$ cells through the activation Ras/MAPK signaling (197).

One of the major gain-of-function properties of mutant p53 is invasion and metastasis. However, whether mutant p53 induced EMT trigger stemness properties in cancer cells, is still quite unexplored. Wild type p53 promotes epithelial differentiation through transcriptional activation of miR-200c (198) which inhibit the translation of EMT activator Zeb1 (**Figure 2**) (199, 200). Zeb1 and Zeb2 in turn repress the other miRNAs of miR-200c family that targets self-renewal factors like Bmi1 (201), and possibly Klf4 and Sox2. Therefore, loss of p53 in mammary epithelial cells leads to a reduced expression of miR-200c thereby promoting EMT and stemness properties and development of a high-grade tumor (198). These observations were corroborated by Pinho *et al.* study in pancreatic acinar cells where they found that loss of p53 leads to increased levels of stemness regulators Bmi1 and Klf4, as well as Vimentin and EMT inducers such as, Snail, Twist, Zeb1, and Zeb2 (88). Although, they did not find any connecting link between the increased stemness and enhanced epithelial to mesenchymal transition phenotype displayed by the p53−/− cells, a high expression of miR-200c can be assumed to be the underlying cause. *TP53* has also been implicated in the suppression of EMT and stemness in the PC-3 prostate cancer cells by modulating the expression of miR-145 (202). PC3 cells expressing wild type p53 were found to express high levels of epithelial marker E-cadherin while the expression of mesenchymal markers fibronectin, vimentin, N-cadherin, and Zeb2 as well as CSC markers such as CD44, Oct4, c-Myc, and Klf4 were reduced. This was rescued upon inhibition of miR-145 in those cells (202). Taken together, *TP53* plays a crucial role in maintaining epithelial phenotype and suppresses pluripotency factors to maintain a differentiated state. However, with the loss of p53 function the suppression on pluripotency genes is lost and this results in activation of EMT and stemness factors. Gain-of function mutant p53 further promotes EMT and stemness phenotypes by activating genes regulating them. For example, in a study by Dong et al., mutant p53 was found to suppress miR-130b expression by binding to its promoter, thereby upregulating the expression of Zeb1, the downstream target of miR-130b (**Figure 2**) (120). Activation of Zeb1 signaling induce Bmi1 expression and promotes stemness (**Figure 2**) (120). Another wild type p53 responsive miRNA, miR-194 has been found to be negatively regulated by mutant p53 in endometrial cancer cells. As miR-194 targets the oncogene Bmi1 which mediates pluripotency, suppression of this miRNA by mutant p53 leads to cancer stemness and EMT phenotypes (**Figure 2**) (120). Mutant p53-R273H has also been found to upregulate lncRNAs, lnc273-31, and lnc273-34 implicated in EMT and

CSC maintenance in colorectal cancer cells (**Figure 2**) (203). Although these studies highlight that mutant p53 mediated EMT phenotype confer stemness in cancer cells, however, there is still a lot to explore in context of molecular mechanisms of mutant p53 driven stemness through activation of EMT genes.

Association With Chemoresistance

One of the major oncogenic gain-of-functions conferred by mutant p53 to the cancer cells is chemoresistance. Mutant p53 singularly regulate a number of pivotal pathways, all of which promote resistance to chemotherapeutic drugs. It is interesting to note that the specific pathways altered by mutant p53 to confer chemoresistance are central to the drug-resistance ability of the CSCs. For example, CSCs abundantly express ABC transporters, that exports drugs out of the cells and imparts chemoresistance (204). Interestingly, one of the important proteins of the ABC family, MDR1, that remains suppressed by wild type p53 in normal cells, is stimulated by mutant p53 in cancer cells during tumorigenesis (**Figure 2**) (80). When normal cells encounter drug induced DNA damage, p53 is stabilized and it triggers cell death by apoptosis. This function is completely lost in mutant p53 cells. In addition, GOF mutant p53 augment the expression of anti-apoptotic proteins Bcl-2 and Bcl-x_L and repress pro-apoptotic proteins Bax, Bad, and Bid (**Figure 2**) (205). In a similar manner, CSCs suppress the Bcl-2 family proteins to attenuate drug-induced cell death (206). DNA-repair mechanisms are mostly impaired in somatic cancer cells. However, CSCs express high levels of DNA-repair genes that helps them repair DNA damage inflicted by chemotherapeutic drugs (207). Murine mesenchymal stem cells (MSCs) with p53 mutations were also found to express high levels homologous recombination repair and non-homologous end joining genes like CSCs (208). Also, mutant p53 expressing iPSCs that induce aggressive tumor in mice, express high levels of detoxifying enzyme associated with drug resistance (15). Despite these similarities there are not many reports on role of CSCs in drug resistance of mutant p53 cells except some indirect ones.

Association With Inflammation and Angiogenesis

GOF mutant p53 can modify the tumor microenvironment and has been found to support chronic inflammation (154). Cancer associated p53 mutants elevate NF-kB activity in response to the cytokine TNF-α and drives cancer progression by elevating inflammatory response (209). Inflammatory response triggered by cytokines has been demonstrated to cause dedifferentiation of cancer cells to CSCs through the activation of various signaling pathways including NF-kB signaling pathway (210). Therefore, it may be presumed that immune response in GOF mutant p53 cells drives cancer stemness by activation of NF-kB pathway. CSCs also exhibit the prominent *gain-of-function* property to induce angiogenesis. Mutant p53 promotes the formation of new blood vessels in tumor by regulating the pro-angiogenic factor VEGF (128). The cancer stem cell niche which supports the long term growth of CSCs, secrete factors that stimulate angiogenesis (211). Moreover, stem cell-like glioma cells (SCLGC) have been found to elevate VEGF to promote angiogenesis (212).

Therefore, it can be surmised that mutant p53 mediated oncogenic *gain-of-functions* potentially drives dedifferentiation of cancer cells to cancer stem cells and *vice-versa* and underlies the enhanced tumorigenesis and poor prognosis of human cancers with p53 mutations.

PROSPECTIVE THERAPEUTIC APPROACHES TARGETING CSCS IN MUTANT p53 TUMORS

Cancer stem cells can arise either from mutations in normal stem/progenitor cells or dedifferentiation of cancer cells (151, 213). Irrespective of the origin, CSCs feature quiescence, ability of self-renewal, therapeutic resistance and metastatic potential (214–216). Loss of wild type p53 function and simultaneous gain of new oncogenic functions by certain missense mutant p53 can generate CSCs or CSC-like features (84, 90, 217–220). Therapeutics that target the intersection between modalities of CSC and p53 mutations are the focus of this section. Many of the discussed therapeutic interventions relevant for targeting CSCs are already in clinical trials in the context of treating mutant p53-based adversities (**Table 1**). Other approaches in restoring wild type p53 functions have been detailed elsewhere (220, 221).

Targeting the Hallmarks of Cancer Pronounced in CSC and p53 Mutant Tumor Cells

Certain hallmarks of cancer like invasion, modified metabolism and proliferation have been found to be active in CSCs as well as p53 mutant tumor cells. Mutant p53 activates SREBP target genes inducing mevalonate pathway that drives cancer cell reprogramming. Mevalonate pathway is lipogenic yielding isoprenoids and cholesterol. Isoprenoids carry out protein prenylation/lipidation and enables proteins like Ras and Rho GTPases to attach with the cell membrane (222). YAP/TAZ, that works through Hippo signaling pathway, induce tissue regeneration, disorganized polarity, CSC features like chemoresistance and metastasis (223–225). YAP/TAZ, together with mutant p53 and NFY transactivate cyclin A, cyclin B and CDK1 promoting cancer growth (226). A functional association among mevalonate enzymes, mutant p53, Rho GTPases, and YAP/TAZ has been implicated (180, 219). SREBP-mevalonate axis is relevant for YAP/TAZ mediated tumor progression. Cholesterol-lowering drug, statins, inhibits HMG-CoA reductase of mevalonate pathway, and blunt YAP/TAZ mediated growth of mutant p53-bearing tumors (**Table 1**) (**Figure 3**) (188). Another instance of metabolic rewiring is the ability of mutant p53 to restrain autophagy by inhibiting AMPK and inducing mTOR pathway thereby ensuring tumor growth (227). In absence of AMPK, mitochondrial stress augments aerobic glycolysis, also called "Warburg effect" in tumor cells, which is promoted by mutant p53 (131). This is potentiated by its tendency of higher glucose uptake aided by mutant p53-mediated increased translocation of glucose transporter GLUT1 to cell membrane (131). Warburg effect is one of the striking

features altered metabolism in CSCs (228, 229). Treatment with antidiabetic drug, metformin (**Figure 3**), and mTOR inhibitor, everolimus, has shown to reduce tumor growth and are being tested in clinical trials (**Table 1**) (230).

In breast cancer cells and mutant p53-KI mouse model of Li-Fraumeni Syndrome, phosphorylation-dependent prolyl-isomerase, Pin1, has shown to augment mutant p53 GOF activities including cellular migration and invasion marked as CSC properties (231, 232). All-trans retinoic acid (ATRA), used in acute promyelocytic leukemia, binds and degrades Pin1 (**Figure 3**) (233). MRX34 is a mimic of miR-34, which can restore the lost tumor suppressor function of mutant p53 (234). Wild type p53 induces miR-34 that can inhibit both tumorigenesis and reprogramming by suppressing myriad genes like like cyclin D1, cyclin E2, CDK4, and CDK6 involved in proliferation; Nanog, N-Myc, SOX2 involved in pluripotency and, SNAIL involved in EMT (235, 236). The phase I study on MRX34 has been recently reported (237). Linc-RNA SOX21-ASI and Linc-RNA HOTAIR can also be important targets as they regulate miR-429 and miR-34a expressions to maintain CSC phenotype (238). Cells bearing mutant p53 depend on G2-M check point for DNA repair, which results from WEE-1 mediated phosphorylation of Tyr15 of Cdk1, inactivating the Cdk1/CyclinB complex required for G2 to M progression (239). WEE-1 inactivation abrogates G2-M checkpoint and drives cells into unscheduled mitosis and death by mitotic lethality (240). The WEE-1 inhibitor, AZD1775 (MK1775), has been included in several clinical trials (**Table 1**) (219). It has been recently found to target CSC properties in breast cancer (241).

p53 Family—An Important Aspect in CSC Regulation

A gain-of-function property of mutant p53 is ability to complex with its family proteins, p63 and p73, which however are not frequently mutated in cancers (242). p53 family members and their isoforms have contrasting effects on differentiation. Wild type p53 and p73 induces differentiation whereas, p63 drives epithelial stem cell proliferation (215, 220). On the other hand, ΔNp73 and ΔNp63 induces enrichment of CSC characters (220, 243, 244). p63 and p73 also play anti-metastatic and pro-apoptotic roles, respectively (114, 245). Mutant p53 can itself disrupt the balance between stem cell proliferation and differentiation as well as sequester p63 or p73 thereby hindering apoptosis, augmenting proliferation, and driving chemoresistance and metastasis typical of cancer stem cells (9, 246–248). Mutant p53–p63 complex can increase RAB coupling protein (RCP)-mediated recycling of cell surface growth-promoting receptors (249). Ras-dependent phosphorylation at Ser6 and Ser9 of mutant p53 forms mtp53-SMAD complex that inhibits p63-mediated anti-metastatic effect (250). Hence, p53 family members present a larger scope of targeting mutant p53-mediated oncogenicity in the context of CSC.

The compound, RETRA disrupts mutant p53-p73 complex restoring p73-dependent transcription and apoptosis (**Figure 3**) (251). Other compounds known to restore effects of wild type p53 in a p73-dependent manner are NSC176327, NSC143491, NSC254681, mTOR inhibitor rapamycin, NSC59984, and prodigiosin (252–254). Short Interfering Mutant p53 Peptides (SIMP) can interact with different mutant p53 proteins and release p73, while peptides aptamers (PA) can inhibit mutant p53 transcription (**Figure 3**) (255).

Therapeutics to Destabilize Mutant p53

Wild type p53 undergoes proteasomal degradation with the help of E3 ubiquitin ligase, MDM2, which in turn is transactivated by wild type p53. However, mutant p53 is unable to transcribe MDM2 causing its cellular stabilization, which is essential for its GOF manifestation (256). Moreover, heat shock protein HSP90 chaperone machinery prevents mutant p53 ubiquitylation and fosters chemoresistance, which is an intrinsic property of CSC (139). Hsp90 stabilizes mutant p53 by inactivating E3 ubiquitin ligases, DM2 and CHIP (257).

Hsp90 can be inhibited by 17AAG or its derivative, 17DMAG, in combination with HDAC inhibitor suberoylanilide hydroxamic acid (SAHA/vorinostat) (**Table 1**, **Figure 3**) (257). Ganetespib is another Hsp90 inhibitor used in similar context (139). Panaxynol is another Hsp90 inhibitor that reportedly targets lung cancer stem cells (258). Bortezomib and carfilzomib are FDA-approved proteasomal inhibitors for treating multiple myeloma (259). However, mutant p53 in cooperation with Nrf2 transactivates proteasome thereby raising resistance in triple negative breast cancer (260). The resistance can be overcome by combination therapy with APR-246, a molecule that can restore native p53 conformation in GOF mutant p53 (221, 260). Stabilization of Nrf2, which regulates cellular antioxidation, has also been linked to chemoresistance in the context of CSC (261).

Poly (ADP Ribose) Polymerase Inhibition—An Elusive Promise?

Mutant p53 sequesters MRE11 hindering ATM-mediated double strand break repair (161, 262). It can complex with E2F4 and

TABLE 1 | Some clinical trials targeting common mechanistic pathways related to both mutant p53 and cancer stem cells.

Product name	Pathways involved	Phase	Status	Clinical trial registration	Link
Statin	mevalonate pathway	Phase 2	recruiting	NCT03358017	clinicaltrials.gov/ct2/show/NCT03358017
Metformin	mTOR pathway	Phase 1	completed	NCT01981525	clinicaltrials.gov/ct2/show/NCT01981525
		Phase 2	recruiting	NCT03047837	clinicaltrials.gov/ct2/show/NCT03047837
		Phase 1	completed	NCT02312661	clinicaltrials.gov/ct2/show/NCT02312661
SAHA or vorinostat	proteasomal degradation	Phase 1	active, non-recruiting	NCT02042989	clinicaltrials.gov/ct2/show/NCT02042989
AZD1775 or MK1775	cell cycle regulation	Phase 2	completed	NCT01357161	clinicaltrials.gov/ct2/show/NCT01357161
		Phase 2	active, non-recruiting	NCT02101775	clinicaltrials.gov/ct2/show/NCT02101775
		Early Phase 1	recruiting	NCT02659241	clinicaltrials.gov/ct2/show/NCT02659241

downregulate homologous recombination factor, BRCA1 and, single strand break repair factor, Rad17 (149). However, it potentiates the replication factors, topoisomerase 1 (Top1), PCNA and MCM4, and the error-prone repair factor, poly(ADP ribose) polymerase 1 (PARP1) (141, 263). This underscores the significance of PARP1 inhibitors (PARPi) to augment synthetic lethality in the context of mutant p53-mediated incapacitation of DNA repair (**Figure 3**) (141, 264). PARPi has been found to induce chemosensitivity in colorectal cancer stem cells (265). However, similar therapy has shown to enrich resistant $CD133^+$ ovarian CSCs by inducing alternative DNA repair based on DNA meiotic recombinase 1 (DMC1) (266).

CONCLUDING REMARKS

Stem cells residing at the apex of tissue hierarchy, self-renew and differentiate to maintain tissue homeostasis and ensure proper development and regeneration. Imbalance between these two processes results in tissue malfunction and formation of tumor. p53 plays a crucial role in maintaining this balance and conserves tissue hierarchy. It also acts a barrier for dedifferentiation and reprogramming and prevents the transformation of somatic cells to stem cells. In response to DNA damage, activated p53 either promotes differentiation or triggers apoptosis, thereby preserving genome integrity of SCs. Loss or *gain-of-function* mutations in *TP53* induce dedifferentiation and proliferation of SCs with damaged DNA leading to the generation of CSCs.

GOF mutant p53 augments malignant transformation by promoting cell proliferation, metastasis, angiogenesis, resistance to cell death and chemotherapeutic drugs. In recent years, GOF mutant p53 has been implicated in promoting somatic cell reprogramming, CSCs formation and expansion. CSCs, the cornerstone for tumor initiation, progression, and relapse share several oncogenic properties with GOF mutant p53 cells. Therefore, it would be interesting to investigate whether these oncogenic phenotypes are conferred by the increased CSC population residing in GOF mutant p53 tumors or *vice-versa*. As CSCs contribute to drug-resistance and subsequent tumor relapse, targeting them may improve the therapeutic efficacy in *TP53*-mutated tumors. Conceptually, drugs that target common pathways operating in mutant p53 cells and CSCs might have better therapeutic efficacy than those that solely target mutant p53. A few such drugs are already in different phases of clinical trial. Further insights into the underlying molecular mechanisms of mutant p53-driven heightened stemness can open up new therapeutic avenues to selectively target aggressive CSCs in *TP53*-mutated human cancers.

AUTHOR CONTRIBUTIONS

The manuscript was designed and conceptualized by DG, DDG, and SR. DG and DDG contributed to the section on wild type p53 and stemness. DG contributed to the section on the effect of p53 inactivation and p53 GOF mutations on stemness. DDG contributed to the section on therapeutic strategies. DG prepared the figures. The manuscript was critically revised by SR. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Amplifying Tumor–Stroma Communication: An Emerging Oncogenic Function of Mutant p53

Valeria Capaci[1,2†], *Fiamma Mantovani*[1,2*] *and Giannino Del Sal*[1,2,3*]

[1] Dipartimento di Scienze della Vita, Università degli Studi di Trieste, Trieste, Italy, [2] Cancer Cell Signalling Unit, International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy, [3] Fondazione Istituto FIRC di Oncologia Molecolare (IFOM), Milan, Italy

TP53 mutations are widespread in human cancers. An expanding body of evidence highlights that, in addition to their manifold cell-intrinsic activities boosting tumor progression, missense p53 mutants enhance the ability of tumor cells to communicate amongst themselves and with the tumor stroma, by affecting both the quality and the quantity of the cancer secretome. In this review, we summarize recent literature demonstrating that mutant p53 enhances the production of growth and angiogenic factors, inflammatory cytokines and chemokines, modulates biochemical and biomechanical properties of the extracellular matrix, reprograms the cell trafficking machinery to enhance secretion and promote recycling of membrane proteins, and affects exosome composition. All these activities contribute to the release of a promalignant secretome with both local and systemic effects, that is key to the ability of mutant p53 to fuel tumor growth and enable metastatic competence. A precise knowledge of the molecular mechanisms underlying the interplay between mutant p53 and the microenvironment is expected to unveil non-invasive biomarkers and actionable targets to blunt tumor aggressiveness.

Keywords: missense mutant p53, tumor microenvironment, cancer secretome, precision therapy, vesicular trafficking

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Virginia Commonwealth University, United States
Nick Clemons,
Peter MacCallum Cancer Centre, Australia

***Correspondence:**
Fiamma Mantovani
fmantovani@units.it
Giannino Del Sal
gdelsal@units.it

†Present address:
Valeria Capaci,
Dipartimento Universitario Clinico di Scienze mediche, chirurgiche e della salute, Università degli Studi di Trieste, Trieste, Italy

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INTRODUCTION

Tumors are dynamic ecosystems undergoing constant evolution. Transformation entails accumulation of genetic and epigenetic changes in tumor cells, as well as paracrine modification of the surrounding tumor microenvironment (TME). The TME is constituted by an extra-cellular matrix (ECM) providing trophic and mechanic support, populated by non-neoplastic cells, including fibroblasts and vascular cells, as well as innate and adaptive immune cells, which may also infiltrate the tumor parenchyma. During tumor development, the TME undergoes progressive reshaping, switching from a tumor-antagonizing function to an increasingly permissive and ultimately supporting role towards cancer progression. This process involves reciprocal shuttling of a variety of signals between transformed and stromal cells. Tumor cells release a plethora of molecules mediating communication amongst themselves. Moreover, they also secrete soluble mediators that activate cells of the tumor vasculature, thus inducing angiogenesis, and coopt stromal

and bone marrow-derived fibroblasts, which remodel the ECM facilitating invasion. Finally, tumor cells recruit and reprogram inflammatory and immune cell populations to support aggressive tumor phenotypes, including immune escape and chemoresistance. In addition to local effects at the primary tumor site, cancer messaging also displays long-range consequences favoring metastatic outgrowth at distant tissues (1–3).

Mutations in oncogenes and tumor suppressors instigate a pro-tumorigenic crosstalk between cancer cells and their microenvironment, acting at the transcriptional level to dictate the composition of the tumor secretome (4–7). Moreover, oncogenic pathways transduce and amplify signals originated by tumor neighborhoods, including oxygen (Hypoxia-Induced Factors HIFs) and nutrient (mTOR) levels, inflammatory messengers (NFκB), and mechanical inputs (YAP/TAZ). The pleiotropic activities of mutant p53 oncoproteins, expressed as a result of missense *TP53* gene mutations (hereby referred to as mut-p53) are exemplary of how oncogenes affect the tumor-stroma crosstalk. Mut-p53 promotes cancer cell invasion, metastasis, and chemoresistance by reprogramming gene expression, regulating metabolic processes and other cell-intrinsic activities [extensively reviewed in (8–10)]. As we shall describe in this review, it is becoming increasingly evident that mut-p53 heavily contributes to these cancer hallmarks also by affecting tumor-stroma communication at multiple levels.

MUT-P53 DICTATES THE COMPOSITION OF THE TUMOR SECRETOME

An increasing body of evidence indicates that mut-p53 promotes the release of soluble mediators (growth factors, cytokines, chemokines), ECM components, remodeling enzymes and exosomes, all of which display autocrine or paracrine activity on tumor and stromal cell populations, hence fostering cancer cell migration and invasion (11–16).

As exhaustively described in a recent review by Stiewe's group (17), p53 mutants coopt various transcription factors (including Ets-1/2, HIFs, NFκB, STATs, SP1, ID1, p63, and p73) and chromatin remodelers (e.g. SWI/SNF and COMPASS) to induce the expression of secreted bioactive molecules (**Figure 1**). A paradigmatic example is the ability, remarkably conserved across different p53 mutants, to exploit the p63 tumor suppressor as a chaperone to tether to its target promoters, driving the expression of a cluster of pro-invasive soluble factors (16). Among these is the secreted protease inhibitor A1AT (alpha-1 antitrypsin), that has been identified as an indispensable effector of mut-p53 in driving EMT and invasion of non-small lung cancer cells, based on the ability of A1AT knockdown and blocking antibodies to attenuate mut-p53 induced cell migration and invasion (15). Consistently, A1AT expression correlated with adverse prognosis in mut-p53 expressing lung adenocarcinoma (15). Mut-p53 R248Q and R282W also coopt p63 to up-regulate miR-155, which targets the transcriptional repressor ZNF652 and thus promotes expression of messengers, receptors, and transducers driving invasion and metastasis, including TGFβ1, TGFβ2, TGFβR2, EGFR, and SMAD2 (18).

The angiogenesis switch represents a crucial cell-extrinsic cancer hallmark, which mut-p53 sustains by several mechanisms. In complex with E2F1, p53 mutants R175H, R273H, and R280K activate transcription of ID4, a member of the ID protein family that stabilizes mRNAs encoding the pro-angiogenic factors IL-8 and CXCL-1 (19). More recently, mut-p53 R175H and R273H were reported to bind ID4 in breast cancer cells, recruiting the lncRNA MALAT1 to modulate the splicing of VEGFA pre-mRNA, thereby promoting the production of pro-angiogenic VEGFA isoforms (20). Expression of mut-p53 in bone marrow stromal cells was shown to increase VEGF synthesis by directly inducing its promoter, as well as *via* activation of PKC (21, 22). It has been observed that fibroblasts harboring R175H or R273H mut-p53 display enhanced expression of various secreted tumor-promoting molecules such as the SDF-1 chemokine, which fosters growth and metastatic spread of co-transplanted tumor cells in mice (23). Paracrine oncogenic properties of stromal mut-p53 may be important for patients of the Li Fraumeni familial cancer predisposition syndrome, who develop tumors embedded in a mut-p53 expressing stroma (24, 25). Notably, Li-Fraumeni fibroblasts expressing mut-p53 R248Q have an increased rate of global secretion as compared to wild-type p53 expressing cells (26), suggesting that *TP53* mutation may induce this pro-tumorigenic phenotype also in pre-neoplastic tissues. In addition, it has been reported that exposure to environmental cues including hypoxia (27) and growth factors (28), as well as inactivation of the Hippo tumor suppressor pathway (29) may turn wild-type-p53 into a mutant-like structural and functional state (**Figure 2**), suggesting that mutant-like p53 activities in non-transformed cells of the TME may have wider prevalence and stronger impact than initially appreciated.

Finally, the ability of mut-p53 to rewire tumor cell metabolism is also expected to affect the release of signaling metabolites. As an example, stimulation of the Warburg effect by mut-p53 (R175H, R248Q or R273H) leads to enhanced secretion of lactate (30), a metabolite that can induce tumor-promoting inflammation and immune suppression by different means [extensively discussed in (11)]. Moreover, when shifted to a mutant-like state, p53 upregulates expression of PTGS2, a key enzyme in biosynthesis of prostaglandin E2 (PGE2), which stimulates angiogenesis and immunosuppression (29). Given the ability of missense mut-p53 to induce the expression of genes belonging to different metabolic branches, including lipid biosynthesis pathways (31, 32), it is conceivable that the messaging secretome released by tumors bearing *TP53* mutations may be enriched in bioactive lipid molecules with signaling functions. Wild-type p53 has been reported to regulate amino acid metabolism (33); similar to other oncogenes, mut-p53 could also lead to an unbalance of cellular availability of amino acids and of their altered flux in the tumor niche (34), potentially influencing the activity of stromal and immune cell populations.

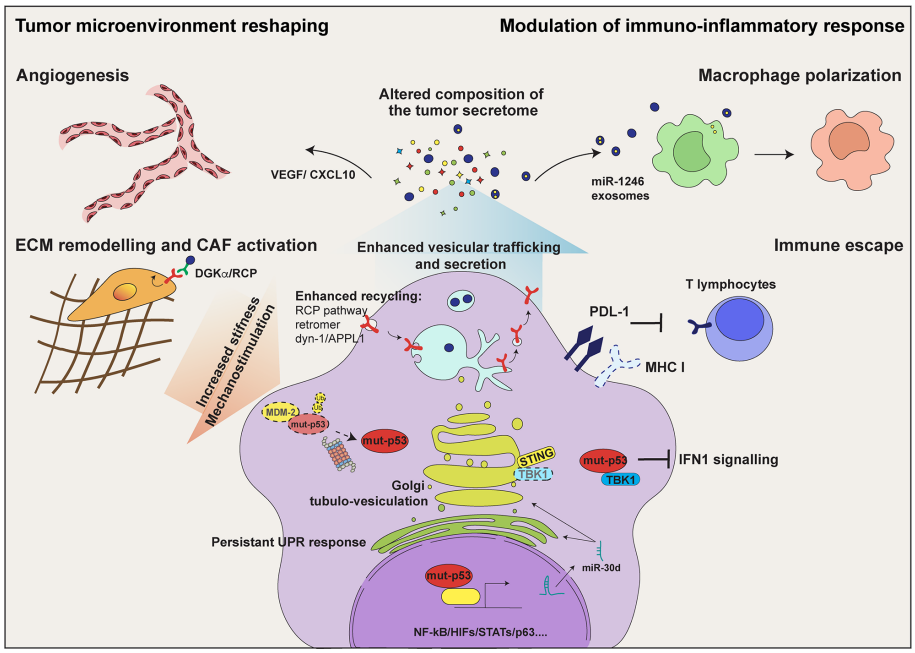

FIGURE 1 | mut-p53 alters the communication of tumor cells with their microenvironment. In tumor cells mut-p53 interacts with a plethora of transcription factors including NF-κB, HIFs, and STATs, and regulates the expression of genes encoding secreted proteins. This activity alters the composition of the tumor secretome and hereby the communication of tumor cells among them and with non-transformed stromal cell populations. Mut-p53 driven secretion of soluble proteins, including cytokines/chemokines and growth factors, induces tumor cell invasion and migration, immune evasion, tumor-promoting inflammation and angiogenesis. In addition, mut-p53 interferes with the function of the cytoplasmic DNA sensing machinery, i.e. cGAS-STING-TBK1 complex, abrogating type I interferon response and disabling the innate immune response. Moreover, mut-p53 stimulates secretion of extracellular matrix (ECM) components and matrix remodeling enzymes, thereby altering the biochemical and biomechanical properties of the ECM and promoting activation of Cancer Associated Fibroblasts (CAFs). This also results in cancer cell mechanostimulation, sustaining the stabilization of mut-p53 protein. By inducing the expression of miR-30d (see text for details), mut-p53 fosters diacylglycerol (DAG) signaling in the Golgi Apparatus, causing morphological and functional alterations known as Golgi Tubulo-Vesiculation, thus enhancing total protein secretion. Persistent ER stress, consequent to enhanced secretion, evokes several mut-p53 activities that support cell survival, including modulation of the unfolded protein response (UPR). By sustaining EGFR and integrin signaling *via* the Rab-coupling protein (RCP) pathway and dynamin-1/APPL1 endosome feedback loop, mut-p53 facilitates cancer cell migration and invasion. Finally, mut-p53 modulates tumor cell messaging also through exosome secretion. The release of podocalyxin-rich (PODXL) exosomes contributes to activate CAFs and promotes ECM remodeling; mut-p53 dependent release of exosomes enriched for miR-1246 induces macrophage polarization towards pro-tumorigenic M2 phenotype, further stoking tumor promoting inflammation.

MUTANT P53 AS A SENSOR OF CANCER-RELATED MICROENVIRONMENTAL CUES

The effectiveness of the p53 tumor suppressor in providing a barrier against neoplastic transformation largely relies on its unique ability to act as a sensitive collector of stress inputs, both intrinsic and extrinsic to incipient tumor cells. Likewise, mut-p53 oncoproteins also respond to cancer-associated stress conditions, including microenvironmental cues (8), and hence their oncogenic activity becomes empowered by a tumor-supportive microenvironment.

During tumor evolution the physical, biochemical, and biomechanical properties of the ECM become altered *via* increased matrix secretion and remodeling, operated by both cancer cells and cancer-associated fibroblasts (CAFs). The resulting increase of matrix rigidity triggers cell mechanotransduction pathways, fostering cancer cell EMT, invasion, dissemination, and chemoresistance, as well as activation of tumor-supporting cancer-associated fibroblasts (35). Mut-p53 has a remarkable ability to induce ECM changes. Matrix metalloproteinases play a critical role in cancer cell invasion and dissemination by degrading ECM proteins. Mut-p53 has been shown to increase MMP secretion and activity, e.g. through upregulating the MMP9 gene (36) and inhibiting the expression of the tissue inhibitor TIMP3 (37). In non-small cell lung cancer (NSCLC) cells, p53 mutants R273H and R246I were shown to cooperate with HIF to upregulate the expression of matrix components, including type VIIa1 collagen and laminin-γ2. This activity associates with increased NSCLC tumor growth in mouse models, and with adverse prognosis of lung cancer patients (38). Accordingly, work by our group demonstrated that in breast cancer cells mut-p53^{R280K}/HIF1α promote ECM deposition and stiffening, thereby sustaining mechano-stimulation and functional activation of CAFs both at primary and secondary tumor sites (26). Importantly, mut-p53 acts as mechanosensitive oncoprotein, being stabilized and activated downstream to actomyosin dynamics induced by ECM rigidity (39). Thus, by increasing ECM stiffness, added to the ability to stimulate cell mechanoresponsiveness *via* activation of integrin

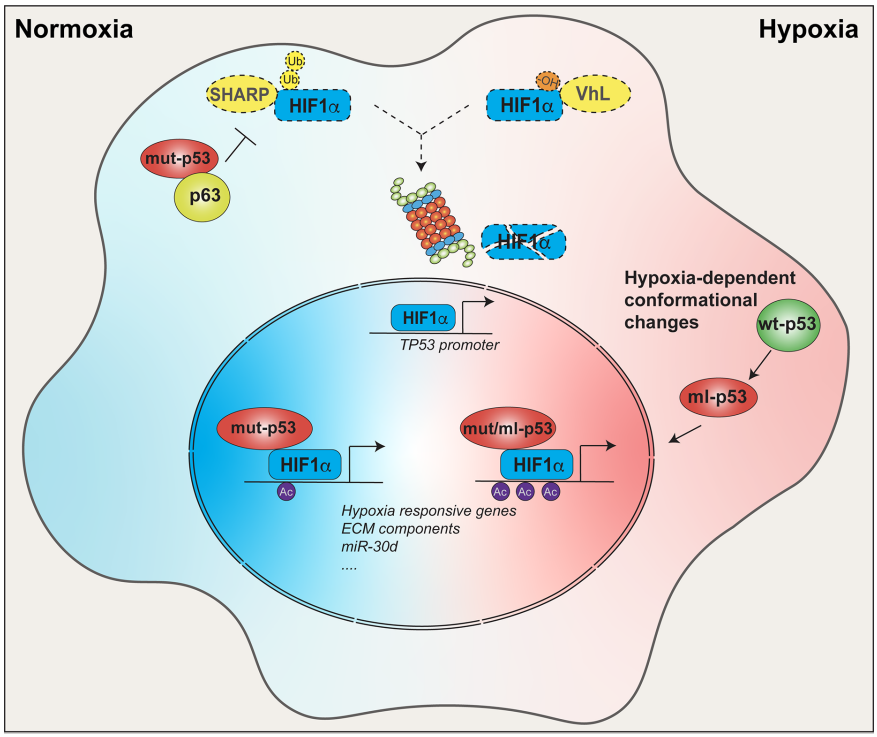

FIGURE 2 | Crosstalk between mut-p53 and HIF1α within tumor tissues. In tumors, mut-p53 sustains HIF1α function in both normoxic and hypoxic conditions. In normoxic conditions, mut-p53 interacts and inhibits the p63 tumor suppressor, thereby abrogating SHARP1-mediated degradation of HIF1α. In hypoxic conditions, HIF1α is stabilized by loss of VHL- dependent ubiquitination independently of p53 status. In both conditions mut-p53 is able to bind and cooperate with HIF1α at the chromatin level to promote expression of hypoxia responsive genes, ECM components (e.g. type VIIa1 collagen and laminin-γ2), miR-30d, and other tumor-promoting HIF1α target genes. Moreover, p53 and HIF1α are connected by a positive feedback loop. Under hypoxia, and likely in other HIF1α stabilizing conditions, HIF-1α upregulates the *TP53* promoter, driving the expression of p53, either wild-type or mutant. Once translated, wild-type p53 may undergo hypoxia-induced conformation changes acquiring a mutant-like (ml) phenotype. Both mut-p53 and ml-p53 bind HIF-1α protecting it from degradation, and chaperone HIF-1α to hypoxia-responsive elements (HREs), finally enhancing HIF1α transcriptional activity.

recycling (40) and RhoA signaling (30, 39, 41), mut-p53 triggers a mechano-stimulatory circuit that both generates and transduces mechanical cues within tumors.

Tumor hypoxia also represents an environmental condition that drives malignant progression by triggering angiogenesis, invasion, metastatic competence, and therapy resistance, largely relying on HIF-dependent transcription. In this context, expression of mut-p53 was found to empower HIF1α oncogenic activities by several means (**Figure 2**). First, it has been shown that mut-p53^{R175H} contributes to stimulate HIF1α stabilization in hypoxic tumors by dissociating it from MDM2 (42), and also inhibits the p63/Sharp-1 axis, which promotes ubiquitin-mediated degradation of HIF1α (43), leading to its accumulation even in normoxic tumors (**Figure 2**). On the other hand, hypoxia-activated HIF1α can directly induce *TP53* promoter, producing a structurally altered wild-type p53 protein that is capable of chaperoning HIF1α at its cognate chromatin sites, upregulating hypoxia-responsive genes (44). Similarly, HIF1α may increase mut-p53 expression, thus fostering a circuit of oncogene cooperation that enhances pro-tumorigenic alteration of the microenvironment. Indeed, missense mut-p53 was found to sit with HIF1α on its target chromatin sites and recruit SWI/SNF chromatin remodelers, leading to hyper-activation of a specific subset of HIF-regulated genes encoding ECM components (38).

In this context we recently added further evidence of the synergism between mut-p53 and HIF1α showing that, through cooperative activation of the *MIR30D* chromatin regulatory region, these oncogenes boost the expression of the onco-miRNA-30d in both hypoxic and normoxic cells (26). miR-30d was previously reported to promote cancer cell migration/invasion and metastasis by modulating several targets, and indeed it is aberrantly expressed in various tumor contexts (45–48). Interestingly, miR-30d also targets p53 mRNA, inhibiting the expression of wild-type p53 (49). Growing evidence supports the idea that loss of wt-p53 in stromal cell populations including CAFs, mesenchymal stem cells, myeloid suppressor cells, and T cells, causes immune escape and sustains tumorigenesis (50). It is worth noting that this miRNA can be secreted in the TME, and may thus inhibit its target genes also in receiving cancer and stromal cells (51). It is tempting to speculate that, through secretion of miR-30d, tumor cells bearing *TP53*

mutations may contribute to ablating wild-type p53 function in the TME by a non-cell autonomous mechanism. Notably, in cancer cells miR-30d appears to have a negligible effect on the expression of mut-p53 (26), likely due to the high stability exhibited by mutant p53 oncoproteins in tumor contexts (52).

MUT-P53 ENHANCES VESICULAR TRAFFICKING AND SECRETION

During progression, tumor cells frequently display increased secretory activity, which associates with structural adaptations of the secretory apparatus, such as expansion of the Golgi network and optimization of vesicular trafficking (53–56), correlating with poor prognosis (57–60). However, the mechanisms responsible for reprogramming the secretory machinery in cancer cells have remained elusive. We recently highlighted that hot-spot missense p53 mutants, *via* miR-30d-dependent regulation of gene expression, induce major structural alterations of secretory pathway components, including endoplasmic reticulum (ER) enlargement, increase of COP-I and COP-II vesicles, microtubule stabilization and Golgi tubulo-vesiculation (26). In fact, proteomic analyses revealed that in addition to changing the protein milieu secreted by tumor cells, mut-p53 enhances the secretion process of cancer cells. This amplifies the effects of the tumor secretome, contributing to ECM remodeling, stromal neo-vascularization, and CAF activation at local and distant sites (26) (**Figure 1**).

Primary tumors release in the bloodstream factors and microvesicles, which can reshape the stroma of distant tissues rendering them more permissive for survival of disseminated tumor cells and growth of secondary lesions (61, 62). This process, known as pre-metastatic niche (PMN) education, is promoted by mut-p53 also by impacting on the exosomes released by primary tumors. In colon cancer cells, different hot-spot missense p53 mutants promote the release of exosomes containing miR-1246, that switches hepatic macrophages to the tumor supportive M2 status, producing IL-10, TGF-β and MMPs (63). Podocalyxin-rich exosomes, released by pancreatic tumors in a mut-p53^{R273H} dependent fashion, activate integrin signaling in receiving lung cells to enhance deposition of a pro-invasive ECM that facilitates the homing of metastatic cells (64).

During cancer progression and particularly in highly secretory tumors, overwhelming ER protein-folding capacity leads to an unbalance of protein folding, secretion, and degradation pathways known as proteostasis, which evokes complex and intertwined stress-response pathways. These include the unfolded protein response (UPR), a tripartite process that leads to stress adaptation by enhancing protein folding and attenuating translation, but can eventually promote apoptosis if the stress is not resolved (65–68). Recent evidence shows that mut-p53 promotes cancer cell survival and even enhances malignant phenotypes by acting on different adaptive mechanisms to guard against proteostasis and counteract detrimental consequences of proteotoxic stress. Indeed, the ability to enhance proteasomal degradation by coopting the NRF2 transcription factor is a mut-p53 core function conserved among different oncogenic mutants (69). By inducing the UDPase ENTPD5, several mut-p53 variants stimulate folding of N-glycosylated proteins in the ER and their transfer to the Golgi (70), which may contribute to relieving ER stress, as well as enhancing the expression of membrane receptors and secretion of extracellular mediators (65). In fact, ENTPD5 has been identified as a crucial mediator of mut-p53 pro-metastatic activity (70). Finally, it was recently shown that p53 mutants R273H, R280K, and M237I can shift the balance between UPR branches, favoring cancer cell survival in face of ER stress. This occurs *via* dampening IRE1α/PERK dependent pro-apoptotic response, while simultaneously promoting ATF6 activation (71).

Trafficking of integral membrane proteins, including growth factor and adhesion receptors, ion channels and antigen presentation complexes, plays crucial roles in oncogenic signaling and sustains tumor aggressive phenotypes (72). In fact, the ability of mut-p53 to regulate endosomal dynamics and promote recycling of membrane proteins has been associated to oncogenic outcomes. Mut-p53 R175H and R273H proteins were shown to facilitate cancer cell migration and invasion by sustaining EGFR and integrin signaling, both via the Rab-coupling protein (RCP) pathway (40) and through amplifying the dynamin-1/APPL1 endosome feedback loop (73). Specifically, mut-p53^{R273H} induces expression of dynamin-1 (Dyn1), an endosomal protein essential for the recruitment and accumulation of the signaling scaffold APPL1 in endosomal membrane. APPL1-rich endosomes localize at the cell periphery and create a signal integration platform that sustains rapid recycling of EGFR, β1-integrins and focal adhesion components. Another example is activation of RhoA/ROCK-dependent cytoskeleton dynamics, by which mut-p53 R175H, R248Q and R273H proteins were shown to promote GLUT-1 trafficking to the plasma membrane, increasing glucose consumption and stimulating the Warburg effect (30). We recently discovered that mut-p53, via miR-30d, downregulates the VPS26B component of the core retromer complex (26) (**Figure 1**). This structure is essential for endosomal dynamics, regulating both recycling to plasma membrane and retrograde trafficking to the trans-Golgi network (74, 75). Retromer defects, induced by mut-p53, could contribute to remodel endosomal membranes and cause mis-sorting of proteins, leading to increased secretion (26, 76, 77).

Interestingly, these abilities are also transmissible to non-transformed cells: by promoting secretion of podocalyxin-rich exosomes, mut-p53 R273H and R175H proteins were found to modulate RCP/DGKα-dependent endosomal recycling in receiving normal fibroblasts that populate the TME of primary and secondary tumor sites, inducing their α5β1 integrin-dependent activation to a cancer associated (CAF) phenotype, increasing tumor invasiveness (64).

All these evidences illustrate how mut-p53, by inducing ample structural modifications of the secretory trafficking machinery, can amplify the range and intensity of the tumor

cell secretome, while also tuning the entire crosstalk of cancer cells with both surrounding and distant microenvironments.

MODULATION OF IMMUNE-INFLAMMATORY RESPONSES BY MUT-P53

Reprogramming secretory trafficking and secretome composition is also key for communication of cancer cells with the immune-inflammatory infiltrate, which is crucial for evading extrinsic anticancer barriers while evoking tumor-promoting outcomes. p53 mutants R175H, R273H, and D281G were shown to coopt the NF-κB transcription factor to produce a plethora of secreted inflammatory chemokines including CXCL5, CXCL8, and CXCL12 (12) that stimulate cell proliferation and motility, thus driving tumor aggressiveness. To enforce NF-κB dependent transcription, several oncogenic p53 mutants activate NF-κB signaling upon TNF-α stimulation, by promoting p65 translocation to the nucleus (13) and inhibiting the tumor suppressor DAB2IP (14). Moreover, in colon cancer cells mut-p53 activates CXCL1 promoter by a NF-κB-independent mechanism (78). In addition, mut-p53 enhances the pro-inflammatory action of IL-1 by suppressing anti-inflammatory interleukin-1 receptor antagonist (sIL-1Ra) (79). Conversely, mut-p53 dampens anti-cancer inflammatory responses. To constrain cancer growth, CAFs actively secrete IFN-β, however in cancer cells harboring *TP53* mutations R175H and R248Q, mut-p53 alleviates this response *via* SOCS1-mediated inhibition of STAT1 phosphorylation, thereby protecting lung carcinoma cells from its anti-tumor effects (80).

The ability of mut-p53 to affect recruitment and activation of both myeloid and T cells has been recently reviewed by Blagih et al. (50). Of note, mut-p53^{R249S} prevents the expression of TAP1 and ERAP-1, important players of MHC-I mediated antigen processing and presentation (81), contributing to cancer immune escape. In lung cancer, this activity is strengthened by mut-p53- dependent induction of co-inhibitory ligands (such as PD-L1), that further constrain T cell activity upon MHC-I peptide recognition, an activity observed for a wide spectrum of missense *TP53* mutations (82).

Very recently, different oncogenic p53 mutant proteins were reported to suppress tumor immune surveillance by interfering with the function of the cGAS-STING-TBK1-IRF3 cytoplasmic DNA sensing machinery. Mut-p53 binds TBK1 and prevents the formation of a trimeric complex between TBK1-STING-IRF3, which is required for activation, nuclear translocation and transcriptional activity of IRF3, and thus abrogates type I interferon response and activation of the innate immune response (83). Loss of wild-type p53 has been shown to instigate aberrant activation of mobile elements and noncoding RNAs, with concomitant induction of immune inflammatory programs (84, 85). In this scenario, missense *TP53* mutations could endow cancer cells with genomic instability via retroelements-induced genome rearrangements, while silencing the consequent suicide immune response.

In sum, mut-p53 can impinge on several aspects of the communication between cancer cells and immune-inflammatory cell populations of the TME, thereby evoking tumor-supporting inflammation, while concomitantly suppressing innate immune signaling and favoring immune evasion.

THERAPEUTIC IMPLICATIONS

Elucidation of the role of mut-p53 as a regulator of the tumor-stroma crosstalk may offer several hubs for tailoring therapeutic approaches to treat tumors bearing *TP53* mutations. The evidence reported in this review suggests that components of mut-p53 induced secretome could represent ideal non-invasive biomarkers of prognosis and response to existing therapies, as well as actionable targets for personalized treatments. Interfering with selected secreted mediators might indeed inhibit the communication between tumors and host tissues, blocking tumor progression, homing of cancer cells or dormancy escape.

Adding on to this concept, preventing mut-p53 from enhancing the release of a malignant secretome could prove highly effective for blocking tumor progression. Suppressing mut-p53 gain of function has been proposed either *via* inducing its destabilization, by treatment with mevalonate pathway inhibitors or HSP90 inhibitors, or through restoration of wild-type p53 functions by small molecules such as PRIMA-1Met/APR-246 (39, 86–88): indeed, these compounds have been proven to normalize the impact of mut-p53 on Golgi structure and secretion (26). IFNβ was found to reduce mutant p53 RNA levels by restricting its RNA stabilizer WIG1, suggesting that mut-p53 positive cancer patients might benefit from IFNβ treatment (80). Another option might be to interfere with alteration of Golgi structure and function induced by mut-p53 and HIF1α oncogenes, e.g. by hitting HIF1α with specific inhibitors (89). Our results in animal models (26) also suggest that anti-miR-30d therapeutics could prove effective to blunt systemic effects of mut-p53-depedent secretion.

Targeting Golgi components has also been realized using a number of small molecules, some of which have provided encouraging results in preclinical studies. However, these compounds either present major pharmacological limitations that restrict their clinical potential (as for the ARF GTPase inhibitor Brefeldin A) (90), or need further studies to refine their selectivity and toxicity. Golgi recompacting drugs represent an attractive option, however their development is still in the early phases (91). Normalization of mechanosignaling by using the Myosin II inhibitor blebbistatin has proved effective to restore compact Golgi morphology in prostate cancer cell lines, however this would be expected to display excessive systemic toxicity *in vivo*. In this respect the use of ROCK inhibitors, previously shown to blunt cancer cell secretion (7), could be more attractive based on their ability to simultaneously block mut-p53 stabilization induced by stromal stiffness (39).

Lastly, increased PD-L1 expression in mut-p53 positive lung cancer (82) may represent a valuable therapeutic window for use of anti–PD-1/PD-L1 immunotherapy. In sum, the functional outcomes of mut-p53 dependent regulation of secretion and trafficking provide an ensemble of druggable processes, which could be targeted by combination therapies.

CONCLUDING REMARKS

n this review, we have summarized evidence of a bivalent interplay of mut-p53 with the TME. From a cell-intrinsic standpoint, mut-p53 senses inputs originated by tumor surroundings and promotes secretory pathway adaptations. In a cell-extrinsic perspective, mut-p53 generates a multitude of output signals, boosting their local and systemic delivery to non-transformed tissues. Recent advances in our knowledge of the underlying molecular mechanisms has disclosed the importance of mut-p53-dependent secretome for enabling metastatic competence.

Clearly, several questions remain open. Similar to cell-autonomous activities of mut-p53, an open issue regards how much the effects on secretome composition may vary according to distinct p53 oncogenic variants and to specific tumor contexts. On the other hand, it appears that the ability to induce structural alterations of the secretory machinery in tumor cells is conserved, at least among hot-spot p53 mutants. Many implications of these structural adaptations remain unexplored: for instance, Golgi dysfunction induced by mut-p53 might cause mis-glycosylation of ECM components, fostering tumor-promoting inflammation and immunosuppression.

Future research along these directions may disclose further layers of complexity in the effects of mut-p53 on the crosstalk between cancer cells and TME. Exploring the impact of mut-p53 on tumor secretion holds great potential to extract biomarkers for prognosis and prediction of treatment response. Thus, it is advisable that validation of markers and therapeutic targets will be actively pursued along the most promising lines of research, with the recommendation that systematic studies are performed to compare different missense mut-p53 variants in multiple cancer types. We project that these activities may provide effective approaches to blunt tumor aggressiveness.

AUTHOR CONTRIBUTIONS

VC, FM, and GDS collected and discussed the material. VC and FM prepared the figures. VC, FM, and GDS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Mutant p53 as a Regulator and Target of Autophagy

Yong Shi, Erik Norberg and Helin Vakifahmetoglu-Norberg *

Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden

One of the most notoriously altered genes in human cancer is the tumor-suppressor *TP53*, which is mutated with high frequency in more cancers than any other tumor suppressor gene. Beyond the loss of wild-type p53 functions, mutations in the *TP53* gene often lead to the expression of full-length proteins with new malignant properties. Among the defined oncogenic functions of mutant p53 is its effect on cell metabolism and autophagy. Due to the importance of autophagy as a stress adaptive response, it is frequently dysfunctional in human cancers. However, the role of p53 is enigmatic in autophagy regulation. While the complex action of the wild-type p53 on autophagy has extensively been described in literature, in this review, we focus on the conceivable role of distinct mutant p53 proteins in regulating different autophagic pathways and further discuss the available evidence suggesting a possible autophagy stimulatory role of mutant p53. Moreover, we describe the involvement of different autophagic pathways in targeting and degrading mutant p53 proteins, exploring the potential strategies of targeting mutant p53 in cancer by autophagy.

Keywords: autophagy, chaperone-mediated autophagy, cancer, mutant p53, *TP53*

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Alfredo Criollo,
University of Chile, Chile

***Correspondence:**
Helin Vakifahmetoglu-Norberg
Helin.norberg@ki.se

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INTRODUCTION

Today, the tumor suppressor protein, p53, is not only known for its *bona fide* function as a transcription factor that controls a network of responsive genes during various cellular stress to ensure genomic stability and fidelity, but also for its key regulatory function in major signaling and metabolic adaptation, beyond preventing tumorigenesis (1–3). Correspondingly, *TP53*, is one of the most notoriously altered genes and tumor-associated p53 mutations are found with high frequency in more human cancers than any other tumor suppressor gene (4, 5). While mutations are found all

Abbreviations: AMPK, AMP-activated protein kinase; CDKN1A, Cyclin Dependent Kinase Inhibitor 1A; Chmp4C, Charged multivesicular body protein 4c; CMA, chaperon-mediated autophagy; DAPK1, Death-Associated Protein Kinase 1; DNM1, Dynamin 1; DRAM1, damage-regulated autophagy modulator 1; EGFR, epidermal growth factor receptor; EMT, Epithelial–mesenchymal transition; ESCRT, endosomal sorting complexes required for transport; GOF, gain of function; HIF-1, hypoxia inducible factor 1; IGF-BP3, Insulin-like growth factor-binding protein 3; IGFR, Insulin-like growth factor receptor; LAMP-2A, lysosome-associated membrane protein 2A; LKB1, liver kinase B1; MDM2, murine double minute 2; MKK3, Mitogen-activated protein kinase kinase 3; mTOR, mechanistic target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PKM2, pyruvate kinase isoform M2; RB1CC1, Retinoblastoma coiled coil protein 1; REDD1, protein regulated in development and DNA damage responses 1; ROS, reactive oxygen species; SREBP1, sterol regulatory element-binding protein 1; TGFBR, transforming growth factor-beta receptor; TIGAR, TP53-induced glycolysis and apoptosis regulator; TSAP6, tumor suppressor-activated pathway 6; TSC, Tuberous Sclerosis Complex.

over the *TP53* gene (5), most common alterations rise from single-base protein-altering substitutions in the coding region with heavy mutational pressure of particular nucleotides (6, 7). These commonly occurring missense mutations cluster in the DNA-binding domain with often diminished ability to bind specific DNA recognition sequences (2). Consequently, the primary outcome of *TP53* mutations is loss of the wild-type ability to transactivate canonical p53 target genes, which provides a fundamental advantage for cancer development. A good example of this is the mutant $p53^{R248}$, which amplifies the pro-survival effects of wild-type protein by maintaining the expression of *CDKN1A* gene, resulting in an ability to survive glutamine and serine starvation, while this mutant no longer is able to induce cell death or senescence (8). Furthermore, unlike mutations in other tumor suppressors, the vast majority of *TP53* missense mutations result in expression of stable, full-length mutant variants where cancer cells acquire selective advantages by retaining these form of the protein (9, 10). Beyond exerting dominant repression over the wild-type counterpart due to loss of heterozygosity, some mutants might exert new malignant abilities distinct from those simply caused by the loss of the wild-type function (11, 12). Such phenotypes, described as mutant p53 gain of oncogenic function(s) (GOFs) (9, 13), include increased cell proliferation, migration and invasion as well as anti-apoptotic functions, which actively contribute to various stages of tumor progression (9, 14–17). This has led to the assumption that, during development of certain tumor types, mutations leading to the expression of missense proteins appears prone to be selected for over the null mutations. In support of this, patients carrying tumors with mutant p53 proteins display higher oncogenic potential, poor prognosis, poor response to chemotherapy and accelerated tumor recurrence compared to patients with p53 null tumors (13, 14). The enhanced oncogenic GOF potential of p53 mutants, beyond the loss of p53 function, is best exemplified by studies using mice with point mutations (p53R270H/- and p53R172H/) as models for the human Li-Fraumeni syndrome, which is an autosomal dominant inherited cancer susceptibility disorder resulting from germline mutations in the *TP53* gene (14, 18–21). These studies have demonstrated that knock-in mice of mutants corresponding to human R175H and R273H develop distinct tumor spectra with high frequency of metastasis, contrary to that observed in mice with p53 deletion, signifying the gain of function of the mutant p53 proteins.

Since the discovery of oncogenic feature of mutant p53 proteins, there has been a steady increase in the number of described diverse GOFs in many cancer types. This has led to reported phenotypic characteristics, culminating in several mechanisms suggested as basis for the gained mutant specific activities (4, 22, 23). A well-recognized mechanism of gained mutant p53 function is its interaction with other transcription factors (4, 24), causing profound alterations in the cancer cell transcriptome and the resulting proteome. However, there is no consensus on the molecular definition of most aspects of mutant p53 GOF(s) and their consequential effects. Distinct mutation type-dependent oncogenic activities still remain to be defined. In addition, recent methodological advances, such as integrated '-omics' and p53 saturation mutagenesis screens, present new insights into the clinical outcomes in patients with myeloid malignancies, where no evidence of GOF for *TP53* missense mutation could be found. Instead clonal selection, driven by the loss of canonical p53 function or the dominant-negative effect that reduced the tumor suppressor activity of wild-type p53, was suggested as the most prominent factor in the selective advantage associated with p53 mutations (25, 26). These observations argue for a fitness advantage in certain human tumors harboring missense mutations rather than the acquirement of additional functions, suggesting that GOF may be context and tumor type dependent.

It is further important to consider that although few missense substitutions (Arg175, Gly245, Arg248, Arg273, and Arg282) account for about 30% of all *TP53* mutations, there are more than 1,500 types of p53 mutations reported in various cancer types (http://p53.iarc.fr/), and different mutant variants are frequently detected in different human cancers (6). Yet, not all mutant proteins accumulate at high levels in tumor cells, although such stabilization seems key for mutant p53 proteins to orchestrate its oncogenic behavior (2, 27). Further, growing evidence from *in vitro* studies as well as animal models signifies that the oncogenic activities of mutant p53 variants are heterogeneous and can vary with the tissue type and the genetic background of the cells (28, 29). This predicts that tissue-selective mutational activity would manifest as tissue-selective enrichment of select *TP53* mutations. In fact, 25 different *TP53* mutation were found to be overrepresented in specific tumor types (26). Accordingly, it has become evident that not all p53 mutants are equal or behave alike and the prognostic impact of *TP53* mutations are diverse (30, 31). Therefore, generalizations about mutant p53 may not be relevant. Instead, the discrepancy of the type of mutant is important, not only as a conceptual distinction for their unique oncogenic abilities, but also for the clinical implications including diagnosis, surveillance and therapy.

For the purpose of this review, we will focus on describing and discussing the considerable distinct effects of mutant p53 proteins may exert on autophagy, although other mutant p53 activities may affect different aspects of tumor biology. Autophagy is a fundamental catabolic process by which eukaryotic cells digest macromolecules and damaged organelles in the lysosomes. A well-defined positive regulatory role of wild-type p53 on autophagy with resulting counteracting autophagy inhibitory effect caused by *TP53* mutations, is widely recognized and beyond the scope of this review. Instead, the central focus will be on what we currently know about the conceivable roles of distinct mutant p53 proteins in regulating different autophagic pathways. Further, given that alterations in autophagy activity might vary in different types and stages of tumors, we will elaborate on the emerging rationale that the functional effects of distinct mutant p53 proteins on autophagy may also differ. Given that autophagy is tightly connected to dynamic changes in metabolism, we discuss the concept that in certain conditions cancer cells with mutant p53 may favor instead of counteract

autophagy. Furthermore, it is well known today that autophagic pathways are reported to mediate the stability of mutant p53 proteins. In the following parts, we will describe the involvement of different autophagic pathways in controlling the cellular level and degradation of mutant p53 proteins as well as the potential therapeutic strategies for targeting mutant p53 in cancer by various autophagic pathways.

AUTOPHAGY-LYSOSOMAL PATHWAYS

Autophagy is a highly conserved homeostatic recycling process, where it functions to mediate the degradation of cellular macromolecules, damaged organelles or internalized pathogens in the lysosomes (32, 33). Under normal physiological conditions, autophagy is maintained at basal level, however, by responding to perturbations in the extracellular environment, e.g. when encountering nutrient deficiency, cells tune the autophagic flux to meet intracellular metabolic demands (34). Thus, beyond the fundamental significance for cellular quality control purposes and the maintenance of cellular and organismal homeostasis, activation of autophagy provides cells with cytoprotective and metabolic adaptations under stress (33–37). Its timely regulation is, therefore, finely controlled by numerous proteins. Dysregulation of autophagy with subsequent altered protein degradation and cellular metabolism, has severe consequences related to several pathophysiological conditions, such as cancer, infection, autoimmunity, inflammatory diseases, neurodegeneration and aging (38).

Multiple routes of degradation through autophagy coexist in mammalian cells that differ in the delivery mechanisms and target specificity, but converge on the same degradation site - the lysosomes (39). Beyond macroautophagy (MA), usually referred to as autophagy, which is the most extensively studied and well characterized type (39), micro- (MI) and chaperone-mediated autophagy (CMA) pathways, are key components of the cellular machinery that play important roles for lysosome-mediated protein degradation (40–42). MA is a multistep process with a nonselective seizing of cytosolic cargo or in a selective fashion that vary in target specificity and induction conditions. It involves the sequential formation of a double-membrane structure, the phagophore that ultimately fuses with lysosomes to degrade sequestered cargos *via* the activity of hydrolases in autolysosomes (43, 44). While Autophagy-related (Atg) proteins act on the *de novo* synthesis and accompanying elongation and closure of the autophagosomes that engulf the cytosolic cargo during MA (45, 46), MI involves the direct uptake of cargo material by the lysosomal or vacuolar membrane and is suggested to occur by either lysosomal protrusion, invagination or with endosomal invagination (41, 47). CMA, on the other hand, applies to select proteins with a pentapeptide motif related to KFERQ that is recognized by the heat shock cognate 71 kDa protein (Hsc70 (also known as HSPA8)) and co-chaperones (48). This interaction forms a chaperone complex that enables the translocation of the cargo protein into the lysosomal lumen *via* binding the lysosomal receptor, lysosome-associated membrane protein 2A (LAMP-2A) (49).

Regardless of the delivery system, the cargo of the autophagic pathways are digested by the lysosomal hydrolases and engendered building blocks are shuttled back to be reused for biosynthesis of macromolecules (50). In this way, autophagy acts as an important internal source of cellular energy through self-degradation process. Hence, the engagement of autophagic pathways confer stress resistance and sustain cell survival that benefit tumor cell growth, especially in nutrient scarce or hypoxic conditions (51). Furthermore, MA may play a critical role in tumor microenvironment and has been proposed to promote tumor dormancy (52), where cancer cells remain in a quiescent state with the potential to relapse. Consequently, autophagy is exploited by cancer cells and malignant tissues often exhibit altered MA activity (53–55), displaying autophagy addiction to sustain stress resistance. Therefore, inhibition of autophagic flux after induction of pro-survival autophagy has been suggested as a strategy to sensitize multiple human cancer types to chemotherapy. However, the role of MA in carcinogenesis is context dependent with reports indicating both pro-tumorigenic and tumor-suppressive roles (56). As a tumor-suppressing mechanism in early-stage carcinogenesis, autophagy dampens inflammation and promotes genomic stability (57). The direct evidence comes from studies using mouse models with genetic knockout of canonical autophagy-related genes, including *ATG5*, *ATG7*, and *BECN1* where impaired autophagy accelerates tumorigenesis in animals (58). However, once a tumor has been established, the nature of autophagy switches and many aggressive tumors acquire reliance on autophagy for growth and survival (51, 59). Thus, in spite of the dual role of autophagy in cancer development and progression differs depending on the genetic context, type of cancer and tumor stage, it is well established today that autophagy is frequently altered in human cancers, with its activation regarded as one of the characteristic key features that contributing to malignant development. In fact, the limited penetrance of mutations in most autophagy genes across human tumors indicates that many human cancer types preserve autophagy function (60), where several well-established oncoproteins and tumor-suppressors whose depletion or mutation promote tumor formation have emerged as eminent regulators of autophagy. In addition, accumulating evidence now also supports a regulatory role for selective autophagy, including mitophagy and non-MA pathways, in human cancer (61, 62). Although CMA was initially suggested to display pro-tumorigenic functions (63), anti-tumor role for CMA is also proposed under physiological conditions in non-transformed cells (61, 64). Further, subsequent studies have demonstrated that CMA plays a more complex and context-dependent role, where cancer cells from different tissues and tumor stages may display varying CMA activity (62). Moreover, growing number of studies provide new insight as to how increased CMA activity can be beneficial for promoting the degradation of proteins displaying dominant oncogenic pro-survival activities in cancer cells (37, 65–68). However, few studies are conducted to assess

the therapeutic impact of CMA activation in cancer, thus CMA-based treatment options in humans remain speculative. This is mainly due to the lack of potent chemical modulators of this process and limitation in functional CMA analysis, which mainly rely on expression levels of the known CMA component, LAMP-2A. Accordingly, while defining the major cancer-related pathways, beyond oncogenic signaling that affect autophagy and control tumorigenesis is important, the regulation and roles of selective and non-MA autophagy, such as CMA and MI, in cancer still needs further investigation, thus the subject of this review will be mainly focused on mutant p53 and MA.

WILD-TYPE P53 – DUAL ROLE IN MACROAUTOPHAGY REGULATION

In the past decades, the mechanisms governing regulation of autophagy has been intensively investigated and the impact of p53, mainly on MA, is well described by several groups with detailed mechanisms uncovered. Collectively, the action of wild-type p53 as a pro-autophagic factor in human cancer cells is reflected by its transcriptional activity on a wide range of downstream target genes with autophagy regulatory effects that diverge on cellular functions, including; a) stimulating the nutrient energy sensor AMPK (AMP-activated protein kinase) (AMPK β1/β2 subunits, Sestrin1/2) (69, 70), b) inhibiting the signaling of mTOR (mechanistic target of rapamycin) (*TSC2*, *IGF-BP3*, *REDD1*) (71, 72), c) suppression of PI3K (phosphatidylinositol-3-kinase) activity (*PTEN*), d) promoting the expression of the MA core machinery (*ULK1*, *ULK2*, *ATG7*) (73, 74), e) transactivating *DRAM1* (damage-regulated autophagy modulator 1) and splice variants that effects several stages of autophagy (75), f) upregulation of *HIF-1* (hypoxia inducible factor 1), g) inducing regulators (Isg20L1 and HSF1 (heat shock transcription factor 1) that in turn transactivates autophagy related genes (*ATG7*) (76), h) induction of *TGM2* (transglutaminase 2) which promotes autophagic flux by enhancing autophagic protein degradation and autolysosome clearance (77), i) interfering with the inhibitory interactions between Beclin-1 and Bcl-2 family proteins (incl. Bcl2/Bcl-XL, Bad, Bax, BNip, Mcl-1, Puma) by their direct transcriptional up or down regulation or through DAPK1 (Death-Associated Protein Kinase 1) activation, alternatively by DAPK1 mediated MAP1B interaction (78), and direct physically interacting with Bcl-XL and the p53-regulated human tumor suppressor protein p14ARF [detailed reviewed in (79, 80)]. These pro-autophagic functions of wild-type p53 are most likely credited to its tumor suppressor role under conditions of hypoxia, starvation or DNA damage, by which induction of MA assists to cope with different kind of cellular stress to prevent cell damage and maintain cellular integrity. This is further in line with the involvement of wild type p53 in several signaling pathways that promote autophagy, including MAPK (mitogen-activated protein kinase) family proteins, such as ERK (extracellular signal-related kinase) and JNK (c-Jun N-terminal kinase).

However, beyond these established pro-autophagic functions, wild-type p53 can also counteract autophagy. This inhibitory role is often attributed to the cytosolic pool of p53 under normal growth conditions, connected to G0/G1 phases of the cell cycle, and shown to be mediated through both transcription-dependent, but mainly -independent manner, involving; a) inhibition of the AMP-dependent kinase and thereby activating mTOR (81), b) induction of TIGAR (TP53-induced glycolysis and apoptosis regulator) regulating glycolysis and cellular ROS levels (82), c) transcriptional regulation of micro RNAs (miRs) (*miR-34a* and *miRs-34a/34c-5p* that targets *ATG9A* and *ATG4B*, respectively) (83, 84), d) interaction with Beclin-1 that subsequently facilitates its ubiquitination and proteasome-mediated degradation (85, 86), and e) direct molecular association with RB1CC1/FIP200, a mammalian protein homologous to Atg17 (87), and f) by reducing the accumulation of double stranded RNA and activation of PKR (protein kinase RNA-activated) (88).

Conclusively, these observations have led to the current notion that the action of wild-type p53 on MA is complex, highly context dependent, dictated by the cellular microenvironment and stress condition, along with the cell cycle progression and subcellular distribution of p53 that exert dual roles in autophagy regulation. In support of this, cumulative evidence shows that nuclear wild-type p53 can promote mitophagy by transactivation of *PRKN* (Parkin), a key effector of this selective autophagy, involved in degradation of impaired mitochondria (89). Cytosolic p53, on the other hand, inhibits mitophagy *via* direct binding to Parkin, preventing its translocation to the damaged mitochondria that cannot be removed by mitophagy (89). Accordingly, these findings are not only confirmative of the counteractive roles of p53 in autophagy regulation, but also indicative of the involvement of p53 in other autophagy pathways, beyond MA. However, despite the studies exemplified above, there are still important pending questions about the detailed molecular mechanisms that govern the role of p53 in MA. Further, the potential contribution, regulatory role, and the physiological importance of p53 in other selective macroautophagy, microautophagy or CMA is yet to be explored.

MUTANT P53 AS A REGULATOR OF AUTOPHAGY

The Effect of p53 Mutant Proteins on Autophagy

Given that impairment of the wild-type function with predominant pro-autophagic role is provoked by *TP53* mutations, it is expected that mutant proteins can reshape the wild-type-mediated outcomes on autophagy. Accordingly, the current accepted view is that mutant p53 displays a suppressive role in autophagy. This was initially illustrated by the assessment of the effect of ectopically overexpressing 22 different p53 mutant variants on the autophagy in p53 null colon cancer cells (90). Reintroduction of some p53 mutants, including $p53^{A161T,\ S227R,\ E258K,\ R273H/L,R273L}$, but not the $p53^{P151H,R282W}$, exhibited high correlation with efficient

suppressive capacity on basal MA. However, the expression of other mutants, including $p53^{P98S,K120D,V143A,R175C,R175D,R175H,R175P,\ R181H,L194F,S227K,G245C,R248L,R248W,\ R249S,R280K}$, displayed no or less suppressive effects, or in some cases even enhanced MA. This led to the awareness that certain p53 mutants may exert negative effects on autophagy. A shared feature of these mutants, including $p53^{A161T,\ S227R,\ E258K,\ R273H/L,R273L}$, was shown to be their cytoplasmic localization, most likely with a loss-of-function to promote transactivation-dependent stimulation of autophagy (90). In support of this, it was later shown that the $p53^{R175H}$ or $p53^{R273H}$ mutants indeed suppress the formation of autophagic vesicles and their fusion with lysosomes through the transcriptional repression of key downstream p53 responsive autophagy related genes, as *BECN1*, *DRAM1*, *ATG12*, as well as *TSC2*, *SESN1/2* and *P-AMPK*, resulting in the autophagy blockage (91, 92). Correspondingly, the knockdown of these mutants in cancer cells cause augmented autophagy by affecting signaling at various phases of the autophagic process with a concomitant stimulation of mTOR signaling. However, it should be noted that both p53 deletion and missense mutations can substantially affect the mTOR signaling, where an elevated association of Rheb with lysosomal membranes promote active mTORC1 complexes (92).

The autophagy inhibitory role of mutant p53 proteins was further ascribed to transcriptional-independent actions. Some p53 mutants, as $p53^{R175H,L194F,\ R273H}$, were unable to form complexes with endogenous Bcl-2 or Bcl-XL, unlike the wild-type. This loss-of wild type function abolishes the capacity to interact, thus cancer cells bearing mutant p53 sustain the inhibitory interactions between Beclin-1 and Bcl-2 family proteins (93). Further, through mTOR stimulation, the aforementioned mutants also convey negative effects on Beclin-1 expression and phosphorylation, thus suppress the functionality of Beclin-1 in autophagy. Likewise, less directly through mTOR stimulation, the $p53^{G199V}$ mutant was demonstrated to gain regulatory function on STAT3 phosphorylation (94), with subsequent transcriptional activation of HIF-1 suggested to contribute to autophagy inhibition. In fact, several multiple mechanisms by which mutant p53 can stimulate HIF-1 have been identified. These includes increased cellular reactive oxygen species (ROS), resulting from less efficient oxidative phosphorylation, or by interference with the binding of HIF-1α to the ubiquitin-protein ligase Mdm2 in hypoxic conditions. However, the functional role of HIF-1 and hypoxia-related genes in autophagy regulation awaits further investigation.

Moreover, by engaging in protein-protein interactions with other transcription factors as a GOF, some cancer-associated p53 mutants were shown with capability of blocking autophagy indirectly by activating several growth factor receptors, such as TGFBR, EGFR, IGFR (95), contributing to sustained active PI3K/Akt/mTOR signaling that subsequently repress autophagy. In breast cancer cells a direct correlation between mutant $p53^{R273H}$ and Akt phosphorylation was demonstrated. Akt, in turn, propagates the effect on its direct downstream target mTOR. Taken together, regulation of the mTOR activity by either constitutive blockage of AMPK signaling or through alternative routes, appears to represent a crucial signaling that occur in cancer cells bearing mutant p53 (91, 92). Thus, regardless of the transcriptional dysregulation or GOF mediated protein-protein interaction, an important implication of these findings is that the autophagy suppressive role of mutant p53 seems mainly to merge on the canonical AMPK-mTOR signaling.

The Impact of p53 Mutants on Autophagy Through Metabolic Changes

A defining hallmark of cancer is uncontrolled cell proliferation, which is initiated once cells have accumulated adaptations in pathways that control metabolism and proliferation (96, 97). Metabolism provides the energetic and biosynthetic demands of rapid proliferation. Beyond a high glycolytic activity, the most common metabolic alteration in malignancies, rapidly proliferating cancer cells further display a sustained mitochondrial oxidative phosphorylation, as the tricarboxylic acid (TCA) cycle intermediates are important precursors for the synthesis of amino acids, lipids and nucleotides (96–99).

While, a direct interference of $p53^{R175H,\ R273H}$ mutants on MA can be denoted to LOF transcriptional repression of core autophagy genes (*BECN1*, *ATG12*) (91), most of the mutant p53-mediated autophagy inhibitory evidence stems from studies describing a gained regulatory effect of mutants on cancer metabolism (**Figure 1**). As stated above, autophagy is regulated by a number of effectors strictly interconnected with the metabolism as revealed by the fact that mTOR and AMPK are both master regulators of autophagy and major sensors of the cellular energy status (100). mTOR functions as a key homeostatic regulator of cell growth and orchestrates whether anabolic or catabolic reactions are favored. mTOR complex 1 (mTORC1) manages multiple biosynthetic pathways and promotes cell growth when nutrients are in plentiful supply. These include synthesis of amino acids, proteins and biogenesis of ribosomes (101). AMPK, on the other hand, is a highly conserved sensor of the cellular energy status that is activated upon low intracellular ATP levels. AMPK responds to energy stress by suppressing cell growth and biosynthetic processes, in part through its inhibition of the mTOR (mTORC1) pathway (102, 103). Thus, while p53 deletion and missense mutations can enhance mTOR, emphasizing the functional interplay between AMPK and wild-type p53, some mutants can display effects on the canonical AMPK-mTOR signaling beyond the transcriptional repression. An excellent example highlighting the difference between the wild-type function from null and missense GOF mutations, is the ability of $p53^{R175H,G245C,R282W}$ mutants, displaying a negatively metabolic effects on the AMPK signaling through the direct protein-protein interaction with the AMPKα subunit under conditions of energy stress (104). Now, several p53 mutants ($p53^{P151S,\ E336X}$), but not the wild-type, have been shown to interact with AMPKα through the DNA-binding domain, where mutant p53 disrupt the interaction of AMPKα-LKB1. This causes inhibited AMPKα phosphorylation and suppressed AMPK activity. In addition, a role of $p53^{R273H}$ was demonstrated

Type	Metabolism	Autophagy
Wild-type function  WT p53	Suppress glucose uptake, glycolysis and glycolytic flux	**Induction of autophagy (MA).**
	Induce amino acid catabolism and gluconeogenesis	
	Enhance pentose phosphate pathway flux	Suppression of autophagy (MA).
	Promote oxidative phosphorylation and the TCA cycle (cellular contexts)	
	Regulation of lipid metabolism	
Loss of function (LOF)	Downregulation of gluconeogenesis genes	Suppression of autophagy (MA).
	Reduced oxidative phosphorylation	
Gain of function (GOF)  mutant p53	Enhance glycolysis	**Suppression of autophagy (MA).**
	Inhibition of AMPK Activation	
	Suppress/Promote mitochondrial metabolism (cellular contexts)	
	Stimulation of mevalonate pathway	Induction of autophagy (MA).
	Accumulation of metabolites (Lipids) Upregulation of Lysophosphatidic acid (LPA) signaling	

FIGURE 1 | The metabolic and autophagy effects of wt p53, null (loss of function (LOF)) or gain of function (GOF) of mutant p53 as a table illustration. AMPK, AMP-activated protein kinase; MA, Macroutophagy.

to control the mevalonate pathway (MVP) through the transcriptional modulation of SREBP1, a downstream target of AMPK (105). Furthermore, the $p53^{R175H,\ R273H}$ mutants were described to promote phosphorylation on the pyruvate kinase isoform M2 (PKM2) (106), a key enzyme that catalyzes the conversion of phosphoenolpyruvate (PEP) and ADP to pyruvate and ATP in glycolysis. The phosphorylation (Tyr105) on PKM2 enhances the mTOR signaling. However, these functions seem independent of the subcellular localization of p53 mutants, as mutants with acquired capability to functionally inhibit the AMPK signaling can be found both in the cytoplasm, such as $p53^{P151S}$, or localize exclusively in the nucleus ($p53^{E336X}$), most likely due to the lack of a C-terminal p53 nuclear export signal, whereas the cellular localization of the mutant $p53^{G245C}$ differs with the confluency of the cell culture. Nonetheless, based on its effect on the AMPK-mTOR axis, the cancer related expression of $p53^{R248W,C176S,R273H,R175H,\ R175H}$ mutants are shown to display a gained function of affecting metabolism, thereby inhibit autophagy irrespectively of tissue of origin or prevalence to a subcellular localization (91).

Beyond the AMPK-mTOR signaling, several of the metabolic effects of mutant p53 oppose the metabolic functions commonly acquired by the wild-type protein, including glycolysis, lipid metabolism, the mevalonate pathway, *de novo* serine synthesis, urea cycle and oxidative phosphorylation (107, 108) (**Figure 1**). Thus, it is well known that mutant p53 rewires cancer metabolism (109). For example, wild-type p53 limits glycolysis and induces flux through the pentose phosphate pathway (82), whereas mutant proteins induce metabolic responses that include enhanced glycolysis to support tumor cell growth and proliferation. By promoting glucose uptake, mutant p53 can limit autophagy-dependent energy production. Therefore, any perturbation in cellular metabolism and redox control caused by p53 mutants can affect the autophagic outcome. However, this does not only apply to metabolic adaptation of cancer cells as a loss of function or in terms of enhanced glycolysis. For instance, mutant p53 has been shown to promote the MVP opposite to the wild-type p53, which is required for mutant p53 and Hsp40 interaction facilitating mutant p53 stabilization (110, 111). The MVP is an essential metabolic pathway that produces sterols and isoprenoids including cholesterol for the synthesis of membranes and lipids, as well as signal transduction allowing cancer cells to survive under conditions of matrix detachment (105). This in turn could promote detachment-induced autophagy (112). Further, beyond elevated glycolytic rate in cancer cells, several studies have clearly demonstrated that the majority of tumors similarly possess the capacity to sustain high fuel oxidation and ATP production in mitochondria (96–98, 113, 114). Especially, quiescent and slow proliferating tumor cells with activated MA, rather depend on oxidative phosphorylation for energy supply than glycolysis (98). Depending on the cellular context, mutant p53 have been indicated to both inhibit or promote oxidative phosphorylation (29, 115), and can thereby enhance or suppress autophagy. However, metabolic alterations are also observed in p53 null cells due to loss of wild type p53 function, such as a downregulation of genes that facilitate gluconeogenesis, which is observed in mice with an adipocyte-specific loss of p53 (116), and reduced oxidative phosphorylation in *p53*-null cells (117, 118). Accordingly, our understanding of the involvement of mutant p53 in direct interference of the core autophagy machinery and regulation in cancer cells as well as the detailed associated molecular mechanisms, beyond metabolic modifications, remain incomplete and need to be further assessed in human clinical specimens.

Consideration of a Potential Stimulatory Role for Mutant p53 in Autophagy

It is equally important to note that the MA inhibitory function is not shared among all mutant p53 proteins. Mutant arising from the substitution of lysine in position 382 with arginine, fails to associate with FIP200, and loose the autophagy inhibitory function (87). Moreover, the ectopic expression of $p53^{P151H,R282W}$ was shown not to display any efficient autophagy inhibitory behavior, apart from the fact that some mutants even show enhanced MA activities (90). Given that autophagy can sustain tumor cell metabolism, and mutant p53 can foster adaptations to nutrient deprivation, it is conceivable that certain mutant p53 proteins could therefore function in seemingly unprecedented way to respond to nutrient stress, where certain mutants may support the constitutive high levels of MA to provide selective advantage for cancer cells. Therefore, it is reasonable that some mutant p53 forms may enhance autophagy required to prevent energy crisis and maintain nucleotide pools during starvation in cancer cells caused by hypoxia and nutrition depletion in tumor microenvironment. This could be especially relevant under situations of expansion of tumor mass (**Figure 1**), in which some parts of the tumor starve due to insufficient nutrient availability or lack of vascularization, even when cancer cells promote metabolic pathways to support growth and proliferation. Under these conditions, numerous mechanisms, including autophagy activation and mutant p53 might converge to contribute to preserve cell viability as a supportive response. While this hypothesis remains speculative, a recent immunohistochemical study on 113 colorectal cancer specimens uncovered a significant association between high LC3B expression and mutant p53 protein expression pattern in ~35% of the patients (119). Although the type of p53 mutant remained undisclosed, the fact that a co-expression of LC3B and mutant p53 was tightly linked to aggressiveness is indicative of high rates of autophagy in malignant tumors. This feature was not observed in tumors with null expression. While further investigation is warranted, this finding provides the rationale that even when the wild-type ability to promote autophagy might be hampered by mutations, some mutant proteins enable autophagy activation in tumors. It is likely that certain point mutations may selectively retain some of the wild-type p53 pro-survival functions, including the pro-autophagic activity. An intriguing possibility is also that the pro-autophagic function of mutant p53 might be a transient phenotype under limited periods of nutrient starvation. A comparable example of this possibility is the activation of the cyclin-dependent kinase inhibitor p21 by p53. Although p21 expression generally contributes to the induction of an irreversible proliferative arrest, transient p53-mediated induction of p21 is reversible, allowing cells to re-enter the cell cycle once stress or damage has been resolved (120). Alternatively, the mutant p53-driven autophagy suppressive function might be overridden by additional signaling, mutations or epigenetic changes. For instance, in the context of proteasomal inhibition, cancer cells with mutant $p53^{R273H}$ display activated MA (121). Additionally, activating mutations in *HRAS* or *KRAS* elicit excessive MA, regardless of the presence of mutant p53. In contrast to normal cells, *RAS*-driven cancer cells display remarkably high levels of basal autophagy, and it is well acknowledged that a subset of RAS-driven human cancers shows a reliance on autophagy for their survival (54). Concomitant expression of mutant p53 and oncogenic Ras, leading to cellular transformation, and a crosstalk between Ras and various mutant p53 proteins is well documented. However, in the presence of mutant p53, some *KRAS* bearing tumors are still addicted to autophagy (122), indicative of that mutant p53 may not always inhibit MA (123). Perhaps a particular pathway ultimately predominates over others. While, this remains to be investigated, it was shown that different p53 mutants cooperate with H-Ras in different ways to induce a unique expression pattern of a cancer-related gene signatures (124). For instance, the $p53^{R248Q,\ R273H}$ mutants exhibited the highest level of gene expression by cooperating with NFκB, the $p53^{R175H}$ and $p53^{H179R}$ mutant induced the cancer-related gene signatures by elevating H-Ras activity. By contrast, the $p53^{G245S}$ displayed no effect, further emphasizing the significantly different impact and responses different mutants can exhibit.

In addition, beyond the *in vitro* observation that even a seemingly subtle difference of one amino acid, such as $p53^{R248W}$ versus $p53^{R248Q}$ (125), can have a large impact on the mutant p53 function. Additionally, even the same amino acid substitutions at the same position (R175H) in the p53 protein have been shown to dramatically different phenotypic effects in terms of metabolism (29). Thus, in spite of the fact most studies describe a suppressive role of mutant p53 on autophagy, there is evidence that the contribution of individual mutant p53 on autophagy might differ in a cell or tissue type, context or cancer stage-dependent manner. Considering the tumor progression promoting function of both mutant p53 and autophagy, inhibiting autophagy seems to be counterproductive for advanced tumors. Thus, it is reasonable to think that cancer cells would rather benefit from mutant p53 with enhanced autophagy activation that can serve as a cell survival mechanism during certain conditions, similar to the dual nature of autophagy which confers suppressive role in tumor initiation while aggressive cancers acquire autophagy for growth and survival. One such condition during which mutant p53 may favor instead of counteracting autophagy is Epithelial–mesenchymal transition.

Role of Autophagy in Mutant p53-Driven Epithelial-to-Mesenchymal Transition

One of the major transdifferentiation processes, through which cancer cells develop the ability to invade and disseminate is the Epithelial–mesenchymal transition (EMT) (126, 127). This process facilitates molecular and functional changes as such, cells undergoing EMT become invasive by acquiring characteristics required for cancer cells to adapt to phenotypic changes fostering capability to break out of the primary tumor. Beyond facilitating cancer dissemination, EMT can further contribute to stemness and resistance to therapy (128). However, EMT covers a complex and multifactorial spectrum,

which gives rise to a variety of intermediate cell states (129). Consequently, EMT is now recognized as a dynamic and reversible process, rather than a binary state, that involves tumor microenvironment, cellular heterogeneities, as well as phenotypic plasticity, thus various metabolic reprograming occurs along with the EMT process.

It is well known that the EMT pathway is under the negative regulation of wild type p53, while mutant p53 proteins display oncogenic GOF activities with robust capacity to promote EMT by controlling the TGF-β signaling and by regulating the expression of various pro-EMT-Transcription factors (130, 131). Furthermore, considering that the metastatic potential of cancer cells increases along the EMT process, multitude of key metabolic pathways, including glycolysis, the TCA cycle, lipid and amino acid metabolism, have been attributed to contribute to EMT, tumor aggressiveness and invasiveness (132, 133). Yet, some EMT positive tumors are characterized by low proliferation rate or quiescence (134). Tumor cells with the traits of invasiveness and stemness which have undergone EMT program can manifest features of growth arrest, and cancer cell dormancy (135). The resulting stemness may drive the progression of more aggressive tumors. For instance, the TGF-β induced EMT process is related to a slow proliferation rate and cell cycle arrest in epithelial cells (136). This observation is rather counterintuitive as it is difficult to directly explain how a slow-proliferating population can lead to higher tumorigenicity and how these tumor cells can remain and exit dormancy.

One possible explanation might be that the metabolic changes elicited by mutant p53 are not mutually regulated and unidirectionally controlled in all cancer cells and may differ during the different stages of cancer progression, such as with the capacity to undergo EMT (16, 113, 137). So how could this be connected. First, several molecular mechanisms underlying the involvement of mutant p53 in malignant progression and EMT have been reported, which all converge on expansion of epithelial stem cells and induction of stem cell gene signatures, as well as mesenchymal stem cell-derived features (138–140). This suggests that mutations in *TP53* not only sustain primary tumor formation, but also that mutant p53 can promote the late stage of tumorigenesis, possibly through the acquisition of an invasive ability and stem cell characteristics. Secondly, while mutant p53 have been linked to promote glycolysis through distinct mechanisms (141), emerging data supports the notion that not all mutants display enhanced glycolysis. For example, while the $p53^{R175H,\ R273H}$ mutants, are able to confer enhanced glycolysis in lung cancer cells, the stable expression of $p53^{R175H}$ in human breast epithelial cells displayed considerably different properties, characterized by a markedly lowered glycolytic phenotype (29) (**Figure 2**). These data highlight the fact that the same amino acid substitutions, in the same position of a mutant p53 protein can have dramatically different phenotypic effects in terms of glycolysis. Moreover, breast epithelial cells expressing $p53^{R175H}$ displayed enhanced MA, which predicts the inversely correlation between dampened glycolysis and enhanced autophagy. Thus, while mutant p53-enhanced glucose metabolism can correspondingly suppressed autophagy in proliferating cancer cells, it is reasonable that a reduced glycolysis by mutant p53 can induce autophagy in quiescent cells. Keeping in mind that a rewiring of cellular metabolism appears to precede changes in stemness, these data are supportive with metabolic changes observed in slow proliferating circulating tumor cells, which display higher mitochondrial metabolism rather than glycolysis.

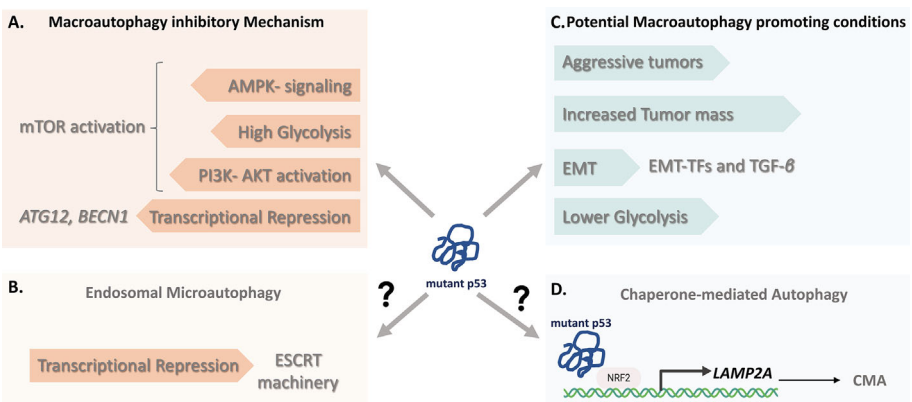

FIGURE 2 | The impact of Mutant p53 on autophagy. **(A)** The macroautophagy (MA) inhibitory mechanisms of some mutant p53 proteins include the resulting transcriptional repression of core autophagy genes (*BECN1*, *ATG12*) and regulation of the mTOR activity by either constitutive blockage of AMP-activated protein kinase (AMPK) signaling or through alternative routes by affecting cancer metabolism. **(B)** Beyond MA, since wild-type p53 can transactivate genes promoting Endosomal Sorting Complex machinery, mutant p53, although yet to be determined, might negatively affect the signaling contributing to ESCRT-dependent mechanism involving endosomal microautophagy. **(C)** Cancer cells bearing some mutant p53 variants may in certain conditions favor instead of counteract autophagy. These include nutrient scarce or hypoxic conditions of aggressive tumors and with increased tumor mass, p53-mediated (EMT situations and hampering glycolysis. **(D)** LAMP-2A expression is an essential factor in CMA activation. Given that transcriptional control of LAMP-2A is shown to be under NRF2, it is likely that mutant p53 proteins might contribute to CMA activation through NRF2-mediated LAMP-2A transactivation, suggesting a molecular connection linking mutant p53 and CMA. CMA, *Chaperone-mediated Autophagy;* EMT, *Epithelial–mesenchymal transition;* ESCRT, *Endosomal Sorting Complex Required for Transport; LAMP-2A, Lysosome-associated membrane protein 2A;* NRF2, *Nuclear factor erythroid 2-related factor 2.*

The observation that tumor cells with mutant $p53^{R72}$ proteins show a greatly increased oxidative phosphorylation as well as increased metastatic ability further supports this (142).

Thirdly, we need to consider the essential role of autophagy induction in supporting cell viability during cancer progression and migration, where MA has clear positive effect on EMT (143, 144). Autophagy induction can be advantageous especially during metabolic reprogramming followed by cancer cell dormancy with a lower proliferation rate or quiescence, thus constitute an efficient adaptive strategy, which can supply of nutrients, confer stress resistance and sustain cell survival during metastatic spreading (145). Taken together, this suggest that mutant p53 may stimulate conditions of metabolic requirement for autophagy induction allowing cells to cope with a stressful or unfavorable microenvironment where cancer cells remain quiescent but may relapse (**Figure 2**). However, it is important to note that data on EMT plasticity and tumor dormancy are primarily derived from *in vitro* studies. Therefore, sophisticated animal studies are needed for tumors that have undergone mutant p53-induced EMT program to provide an *in vivo* correlate in preclinical models. Nevertheless, an important implication of these observations is that p53 mutants do not always acquire and possess the same metabolic consequences and may not display equal biological effects in all types of human cultured cells.

Therefore, when considering the generality of the effect of mutant p53 on autophagy, we might need to keep in mind the metabolic plasticity and different aspects of metabolism might be regulated in different cell or tissue types. The complex regulatory interaction between mutant p53 and autophagy might well be influenced by many factors, such as tissue and cell types, tumor stage, type of other oncogenic mutation, the sequential mutation appearance order, extent of damage or stress, and levels of intra-tumor oxygen or nutrients as well as on the proliferative capacity of the tumor cells. A switch between autophagy phenotypes, depending on fitness landscape or mutation-selection balance may as well applicable for mutant p53 and we need to consider this exceptional plasticity which might create significant challenges as we attempt to therapeutically intervene in these pathways.

The Role of Mutant p53 in Autophagic Pathways Beyond Macroautophagy

To date, there are no direct evidence of a select mutant p53 function in microautophagy, CMA or in selective macroautophagy, including xenophagy, ribophagy. However, as wild type p53 exerts a regulatory role in mitophagy, endosome and exosome biogenesis (146, 147), it is reasonable that mutant p53 proteins might affect undiscovered functions in multiple degradative and cellular sorting systems.

The main limitations of studying non-MA pathways in regards to mutant p53 is likely the incomplete knowledge of their regulatory mechanisms. However, while the signaling mechanisms that control CMA are currently not fully understood, a key step in the CMA process is the expression of LAMP-2A receptor at the lysosomal membrane. High lysosomal LAMP-2A levels are reported to correlate with a predisposition of CMA, whereas silencing of LAMP-2A results in inability to degrade proteins *via* the CMA pathway, thus increase LAMP-2A expression is an essential factor in CMA activation (148). Accordingly, a transcriptional control of LAMP-2A expression is shown to be under the control of the NFE2L2/NRF2 (Nuclear factor erythroid 2-related factor 2 (NRF2) (149), also known as nuclear factor erythroid-derived 2-like 2 (NFE2L2), which generally participates in the control of metabolic redox processes including degradation of oxidized proteins. In 2018, the missense mutant variant $p53^{R280K}$ was demonstrated to interact with NRF2 and to contribute to selective activation of its downstream transcriptional program (150). Thus, given that NRF2 promotes a pro-survival oxidative stress response that allow cells to cope with oxidative stress, along with the fact that CMA is induced by oxidative stress, it is likely that mutant p53 proteins might contribute to CMA activation through NRF2-mediated LAMP-2A transactivation, indicative of a molecular pathway that connects mutant p53 with CMA (**Figure 2**). Consistent with this indication, analysis of various human cancer cells with different mutational p53 status that either expressed wild-type, mutant p53 or null in p53 expression, revealed that Spautin-1 induced CMA in confluent growth conditions selectively induced cell death of mutant p53-expressing cancer cells. No or little effect was detected in wild-type p53 or p53-null cancer cells, suggesting that cancer cells with mutant p53 might be more susceptible to activate or undergo CMA (65).

Moreover, considering that wild-type p53 can transcribe several critical genes encoding endosomal compartment, including *TSAP6*, *CHMP4C* and *CAV1* (147), provides a rationale that p53 signaling may contribute to Endosomal Sorting Complex Required for Transport (ESCRT) machinery dependent mechanism, involving endosomal microautophagy (eMI). However, it is yet to be determined whether there is an involvement of mutant p53 proteins in micro- or endosomal microautophagy (**Figure 2**).

Furthermore, beyond autophagic pathways, mutant $p53^{R273H}$ has been shown to drive alterations in endocytic membrane trafficking during which DNM1 and Myosin VI (Myo6) were upregulated in cancer cells. Apart from stimulating the expression of endosomal proteins, both the wild-type and $p53^{R273H}$ mutants are indicated to effect proteins involved in the secretory pathway, protein secretion *via* extracellular vesicles (EV) and exosomes of endosomal origin (151). Thus, mutant p53 might regulate the expression of components of the endocytic machinery and modify secretion of extracellular vesicles in multiple ways.

MUTANT P53 AS TARGET OF AUTOPHAGY

Targeting Mutant p53 Proteins

Based on the high frequency of *TP53* mutations in human tumors, the oncogenic effects of many missense variants with the fact that cancer-specific pathogenic stabilization of mutant proteins effectively sustains tumor progression and

dissemination, mutant p53 proteins represent indisputable promising targets in cancer therapy (17, 152). Accordingly, different approaches have been explored in which targeting of mutant p53 has primarily focused on the development of therapies designed to inhibit the mutants and restore their wild-type p53 function by small molecules, targeting the gain-of-function phenotype of mutants and stimulating immunological activity directed against a mutant p53 protein (153, 154). In animal models, targeting mutant p53 functions have been shown with highly promising results that selectively kill cancer cells, with low toxicity in healthy tissues, indicating tumor-specific vulnerabilities (105, 155, 156). But in the clinics, the specific targeting of mutant p53 proteins has proven challenging, especially considering that mutations are diverse in their type, sequence context, position, and structural impact, making it difficult to identify a well-defined structure (2, 7). In fact, most desirable oncoprotein targets in cancer therapy, including mutant p53, belong to the intrinsically disordered proteins, which lack a well-defined protein structure making them challenging to pharmacologically target (157). An important factor in anticancer therapeutic failure is also associated with pharmacologic drugs that may lack response to all mutant variant or with substantial toxicity due to loss of wild-type function, or activating wildtype in normal tissue. Thus, while targeting a loss of function is difficult, growing evidence indicate that no single drug may display equal impact on all mutant proteins. Development of different drugs to target distinct mutant p53 or their activities is therefore time consuming and not cost-efficient, although such drugs could make a huge impact. Hence, exploring of alternative approaches to target mutant p53 proteins is therefore of high importance.

During recent years strategies of stimulating the cell's own quality control mechanisms to prevent the aberrant accumulation and induce degradation of oncogenic proteins, including mutant p53, are being explored as a new therapeutic approach. Central to this idea is that oncogenic mutant p53 functions and the mutant p53 addiction of cancer cells is reliant on its sustained high levels, thus this addiction can be therapeutically exploited by targeted mutant p53 degradation strategies. Beyond pharmacological blockade of mutant p53 stabilizing mechanism to promote proteasome-dependent proteolysis, the considerable role for targeted degradation into lysosomes is suggested as a new advance to have a potentially major impact on mutant p53. The targeting of mutant p53 proteins by autophagy activation could offer promising future therapeutic option and is therefore currently investigated intensively. Below, we describe recent advances strategies that and might be potential therapeutic methods.

Targeting Mutant p53 by Macroautophagy

Although mutant p53 proteins were known to accumulate at abnormally high levels in cancer cells, the observation that lysosomal inhibitors could further stabilize mutant protein abundances strongly implied that they might be continuously degraded through the lysosomal pathway. In line with this, glucose restriction in multiple cancer types bearing the $p53^{R175H,\ R280K}$ mutants was shown to induce p53 mutant deacetylation, routing it for degradation *via* MA (158) (**Figure 3**). Accordingly, several studies now demonstrate that lysosomes indeed represent a degradation route for certain mutant p53 proteins (159–161). MA inhibition by either chemical inhibitors or downregulation of key autophagic related genes (*ULK1*, *BCN1* or *ATG5*) induce stabilization of mutant p53, while, the overexpression of Ulk1 or Beclin-1 results in mutant p53 degradation (162). With MA as an emerging important pathway involved in the stability of mutant p53, several classes of small molecules enabling efficient mutant p53 degradation through the induction of autophagy has been described. These include, a) the curcumin-based zinc compound (Zn(II)-curcumin and capsaicin (8-methyl-N-vanillyl-6-noneamide)-induced macroautophagy which have been shown to deplete the expression of $p53^{RH175}$ and $p53^{R273H}$ mutants (159, 163), b) Gambogic acid, a pro-apoptotic molecule that promotes the $p53^{R280K}$ and $p53^{S241F}$ mutant degradation by inducing autophagy (160), c) inhibition of MKK3, a dual protein MAP kinase, which reduces $p53^{R273H}$ mutant protein levels through ER stress-induced autophagy, d) the cruciferous-vegetable-derived phenethyl isothiocyanate (PEITC), which render the $p53^{R175H,R273H,\ R248Q}$ mutants by degradation following reactivation of the mutants, e) heat shock protein 90 (HSP90) inhibitors such as 17-allylamino-17-demethoxygeldanamycin (17-AAG) or ganetespib (155), and f) histone deacetylases inhibitors (HDACi), which have been studied as anticancer compounds based on their potential to stimulate autophagy and to degrade $p53^{R172H,R248Q,\ R280K}$ mutants (155, 164–167). Although apoptosis seems as the main route, inhibiting the HDACs, for example by the suberoylanilide hydroxamic acid (SAHA), a pan HDAC inhibitor, is shown to induce the destabilization of the HDAC6–HSP90–mutp53 complex (165), that results in mutant p53 degradation in cancer cells with pronounced autophagy induction, such as in MDA-MB-231 bearing the mutant $p53^{R280K}$ (164). However, while the MA stimulatory effect of SAHA on cancer cells carrying mutant p53 has been suggested, compared to null or wild-type p53 expressing cells, DLD1 cells carrying the $p53^{S241F}$ allele was not affected by this action. The observed degradation of $p53^{S241F}$ proteins upon SAHA exposure was suggested to relate on alternative degradation pathways rather than MA. Yet, ES2 cell lines bearing the same mutant ($p53^{S241F}$) show difference in SAHA sensitivity. This strongly suggests that, cell type and -contexts need to be considered for SAHA-mediated cytotoxicity in cancer cells. Further, the Zn(II)-curcumin and capsaicin by acting on protein folding is able to reactivate wild-type p53 that induces its target gene DRAM to promote autophagy, while gambogic acid is shown to induce mutant p53 protein degradation through proteasome ubiquitination by carboxy terminus of Hsc70 interacting protein (CHIP). CHIP, on the other hand, known to display chaperone and E3 ligase activity, is involved in stabilizing and degrading both wild-type and mutant p53 proteins, where the degradation of mutant p53 by CHIP was shown to be *via* autophagy through K63-linked polyubiquitination. Under both normal and hypoxic conditions

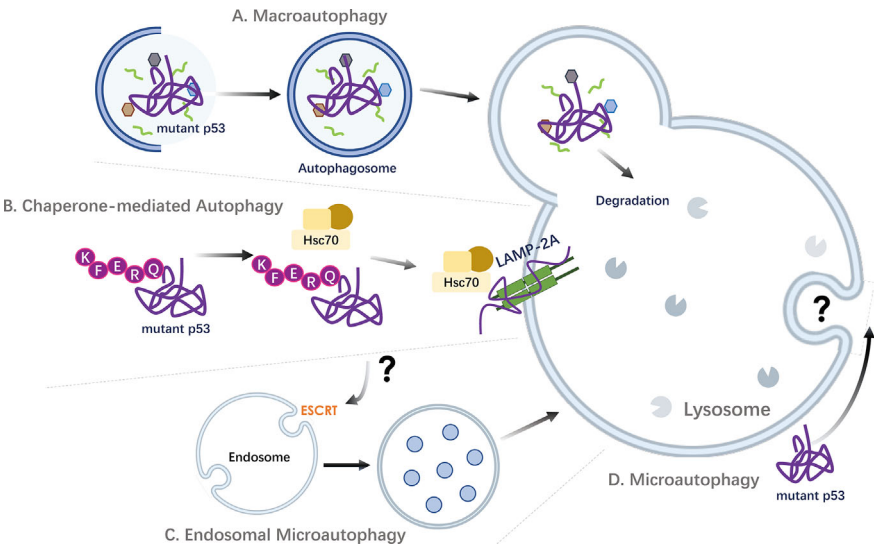

FIGURE 3 | Targeting mutant p53 by autophagic pathways. Strategies of autophagic degradation of accumulated oncogenic mutant p53 proteins in cancer cells. **(A)** Mutant p53 can be engulfed and degraded *via* macroautophagy. P53 containing aggregates have also been implicated to undergo degradation by aggrephagy, a selective sequestration of protein aggregates by macroautophagy. **(B)** As p53 contains KFERQ-like motifs, mutant p53 proteins can be targeted and degraded through the stimulation of Chaperone-mediated Autophagy (CMA). **(C)** *Via* the recognition of proteins harboring KFERQ-like motifs, the molecular chaperone HSC70 and co-chaperone complex can also promote the localization of cargo proteins into endosomal compartments in an ESCRT machinery dependent mechanism, through a process called endosomal microautophagy (eMI). Thus, beyond CMA, other autophagic pathways, including endosomal eMI may mediate the degradation of mutant p53. **(D)** The direct uptake of mutant p53 proteins by lysosomes through microautophagy is not known. ESCRT, *Endosomal Sorting Complex Required for Transport;* Hsc70, *Heat shock cognate 71 kDa protein (also known as HSPA8);* LAMP-2A, *Lysosome-associated membrane protein 2A.*

CHIP was shown to selectively degrade aggregation-prone mutants $p53^{R110L}$, $p53^{R110P}$ and $p53^{R175H}$, without significant effects on the level of nonaggregating mutant $p53^{R248W}$ and $p53^{R273H}$ (168). Moreover, the $p53^{R175}$ degradation by PEITC was reported to be mediated by both the proteasome and autophagy in a concentration-dependent manner, underlying the importance and need for further investigations for the selective degradation mechanism of mutant p53 in order to develop selective autophagy targeting therapeutic strategies. In addition, it is important to note that while the wild type p53 proteins are directed for proteasome-dependent degradation, autophagy-lysosome degradation is also attributed to control cellular p53 stability (169, 170). For example, Sunitinib, a small molecule multi kinase inhibitor, approved for the treatment of metastatic renal cell carcinoma, induced autophagic degradation of wild type p53 proteins in multiple cancer cell lines (169). However, the molecular mechanisms and cellular players involved in autophagic degradation of wild type p53 are still not fully known.

Mutant p53 Proteins as Targets for Chaperone-Mediated Autophagy

Beyond contributing in lysosomal degradation of a select subset of cellular proteins, the discovery of mutant p53 proteins as CMA targets established a regulatory role for CMA in oncoprotein degradation and its potential tumor suppressive role (37, 65, 67, 171). Thus, a new degradative detour for mutant p53 *via* CMA was uncovered (172) (**Figure 3**).

As previously mentioned, CMA is a unique type of mammalian autophagy that only applies to select proteins without targeting cellular organelles (42). Its specificity relies on the recognition of a pentapeptide CMA motif (KFERQ-like) that is a prerequisite in target proteins. The cytosolic heat-shock cognate protein of 70 kDa (Hsc70/HSPA8) plays an essential role in CMA by recognizing the KFERQ-like sequence motifs in substrate proteins. Indeed, p53 harbors two pentapeptide sequences ($_{200}$NLRVE$_{204}$ and $_{341}$FRELN$_{345}$) that are consistent with an Hsc70 recognition motif (65). The FRELN motif is on the linker region, while the $_{200}$NLRVE$_{204}$ motif is exposed on the surface of the p53 protein, making it accessible for recognition.

Once activated, CMA was shown to be very effective in degrading different mutant p53 proteins, regardless of their mutational status (65), including $p53^{P98S,P151H,\ A161T,R175C,\ R175D,R175H,L194F,S227K,S227R,G245C,R248L,R248W,E258K,R273H,R273L,\ R280K,\ R282W}$. This was initially illustrated by the assessment of CMA activation on the ectopically overexpressed above mentioned p53 mutants in a p53 null colon cancer cells. Subsequently, the CMA-mediated degradation of cancer associated endogenous mutant p53 proteins was shown on $p53^{R175H,R248Q,S241F,R158InF,R280L,G266Q}$ variants (65). This suggest that CMA-mediated mutant p53 degradation may be more efficacious than treatment with targeted mutant p53 specific reactivating small molecules and that CMA-based strategy could overcome resistance from acquired mutations. Importantly, the activation of CMA was not or less effective on wild type or p53 null expressing cancer cells. However, contrary

to cancer cells, hepatitis C virus infection induced ER-stress response, which leads to CMA stimulation in untransformed primary human hepatocytes results in degradation of wild type p53 (173). The increased expression of chaperones due to unfolded protein response and ER stress associated with the CMA response, where the genetic silencing of *LAMP-2A* restored the observed p53 degradation. In fact, the silencing of *LAMP-2A* under irradiation conditions was also shown to result in increased p53 protein level (174), in some cancer cells, such as HepG2, which expresses wild type p53 (173). While these studies suggest an interplay between the CMA pathway and wild type p53, it should be kept in mind that p53 interacts with a wide range of different proteins, thus the accumulation of p53 upon LAMP-2A knockdown, may therefore depend on recognition of its molecular partners by the CMA pathway, such as HMGB1 degradation with further impact the wild type p53 protein expression (174). Nonetheless, the discovery that mutant p53 proteins are CMA substrates provided experimental evidence that CMA could be exploited as a novel approach to eliminate mutant p53 in cancer cells. Accumulating evidence now support that CMA activation plays a role in mutant p53 targeting (161). In fact, beyond mutant p53, CMA has been shown to promote the degradation of other oncoproteins, as HK2 and c-Myc (66, 67). Further, a decrease in CMA with age has been associated with higher risk of malignant transformation, and mice with hepatic blockage of CMA has been shown to develop spontaneous tumors (68). While, these findings suggest clinical implications of CMA activation, to date the role of CMA in tumorigenic conditions is not well-defined and there are no direct pharmacological CMA activators for cancer cells. Characterization of such activators would also require that it does not affect other degradation pathways. Accordingly, in order to explore the clinical implementation of CMA, development of applicable methods to measure CMA in live cells, and *in vivo* studies in CMA activation is needed. Thus, to date, there are no clinical studies launched to demonstrate the efficacy of CMA activation in patients. However, the knowledge of the its oncogenic targets, such as mutant p53, and understanding its selective degradation mechanism is an excellent starting point for future development of targeted therapeutic strategies involving CMA.

Mutant p53 as Possible Target for Microautophagy and CASA

Beyond CMA, recognition of proteins harboring a KFERQ-like motif by the molecular chaperone HSC70 can also lead to the endosomal localization in an ESCRT machinery dependent mechanism, through a process called endosomal microautophagy (eMI) (40, 47). Thus, selective degradation of single proteins has been described in a HSC70-driven endosomal eMI pathways (40, 42, 175). In addition, chaperone-assisted lysosomal degradation pathway CASA (chaperone-assisted selective autophagy), has been reported to require the involvement of HSC70. Keeping in mind that the amino acid sequence of p53 contains KFERQ-like motifs that is recognizable by HSC70, although yet to be proven, it is plausible that mutant p53 protein might be targeted by eMI or CASA (**Figure 3**). However, it is currently not known whether wild type and/or mutant p53 proteins are targeted and degraded by these pathways.

Mutant p53 Aggregates as Target for Aggreaphagy

While accumulation of protein aggregates is commonly known for their involvement in the onset of many neurodegenerative diseases, the conformation of mutant p53 with missense mutations is now known to share similarity with that of pathological mutant proteins involved in a wide range of neurodegeneration, including Alzheimer disease, Parkinson disease, and amyotrophic lateral sclerosis, the so-called protein conformation diseases that involve protein misfolding in their etiology (176). Accordingly, mutant p53 proteins display hyperstability due to acquired misfolding and partially denatured conformation with high tendency to form amyloid like micro- and macro-aggregates both *in vitro* and *in vivo* (177). The aggregation of mutant p53 (amyloid oligomers and fibrils) confers a prion-like activity on the native protein, converting it into an inactive form, thus contribute to its oncogenic function (178). The formation of aggregates largely depends on cellular chaperones and chaperone-assisted proteins. Accordingly, mutant p53 stabilization is achieved by the interaction with chaperone heat shock proteins (HSP), including HSP90, HSP40 and HSP70, that cooperate in stabilizing mutant p53.

Aggregated proteins can be degraded by the proteasome or CMA, however, only after the dissolution into soluble single peptide species, unless targeted by a process called aggrephagy, a selective sequestration of protein aggregates by macroautophagy (179). While the molecular mechanism of cargo selection during aggrephagy needs to be further elucidated, $p53^{R175}$ containing aggregates have been implicated to undergo degradation by this pathway (167). This is in fact in line with the observations that CHIP, beyond targeting wild-type p53 by K48 polyubiquitinition, preferentially degrades aggregation-prone mutant p53 proteins through K63 polyubiquitinition chains (168). Thus, although accumulation of mutant p53 occurs only in cancer cells, in which most missense mutants are shown to be more stable than wild-type p53, the aggregation of different mutants seems to correlate with individual structural characteristics, which may affect their differential recognition and degradation route.

Degradation of Mutant p53 Proteins by Multiple Autophagic Pathways

Autophagy pathways are mechanistically and functionally linked such that blockage to either one can lead to upregulation of the other in a way. The degradation of distinct mutant variant can therefore vary between the different types of autophagy, when one pathway is blocked or inhibited, or in response to different stresses. However, it is important to note that although MA and CMA are both operational under normal nutritional conditions, their basal activities are not sufficient for efficient removal of mutant p53. Rather, as described above, mutant proteins can

undergo degradation through MA induced by glucose restriction or by proteasomal inhibition, however when MA is inhibited, which significantly accelerates the activation of CMA that in turn promotes the degradation of mutant p53. This differential degradation route was demonstrated for the $p53^{R248Q}$ mutant in a context dependent manner. In tumors growing in normoxia, with no stress, the treatment with Hsp90 inhibitor (17-AAG) was able to induce the degradation of $p53^{R248Q}$ through MA (161). However, during metabolic stress caused by the pyruvate dehydrogenase kinase-1 (PDK1) inhibitor dichloroacetate (DCA), $p53^{R248Q}$ proteins were stabilized by increased interaction with the Hsp90 chaperone machinery. Thus, in this condition, the co-treatment of 17-AAG instead promotes the association of $p53^{R248Q}$ with Hsc70 and CMA activation, resulting in $p53^{R248Q}$ degradation *via* the CMA pathway (161). Thus, different metabolic contexts and stressors induce diverse autophagy mechanisms that can degrade mutant proteins. In fact, beyond enabling efficient $p53^{R248Q}$ degradation by either MA or CMA, the HSP90 inhibitor, geldanamycin, has been suggested with an unspecific ability to activate CMA.

Furthermore, it is unclear whether different autophagic pathways may display any preference to degrade certain mutants. Since mutant p53 proteins encoded by different mutant alleles exhibit a distinctive tendency to misfold and aggregate, it may affect their susceptibility for recognition and targetability, thus it is reasonable that the mutational status may play a determinant role in its ability to be degraded through the distinct autophagic system. For instance, this may be due to the diverse ability of certain mutant to aggregates into prion-like amyloid oligomers, including $p53^{R175H,\ R249S}$, which can form larger multimeric assemblies, while $p53^{R248Q}$ mutant displays significantly increased amyloidogenic potential, whereas $p53^{M237I}$ mutant is shown to co-localize with amyloid oligomers (180). Thus, beyond defects in degradation and recognition mechanisms, the accumulation of mutant p53 proteins to different levels in cancer cells may depend on their targetability by multiple vs certain degradation pathways.

CONCLUDING REMARKS

To conclude, the role and impact of mutant p53 in autophagy regulation is complex, context-dependent and far from fully elucidated. Growing evidence along with rapidly developing genome editing and omics techniques are likely to revolutionize new roles and autophagic activities of different mutant p53 proteins that may vary according to changes within tumors or in the tumor microenvironment. These new technologies may shed new insights for a knowledge-based discovery to identify knowledge gaps and analyze scenarios that require a reconsideration for the function of mutant p53 on autophagy.

Further, since it is clearly demonstrated that mutant p53 stabilization is a tumor-specific vulnerability, strategies to promote the degradation of mutant p53 by autophagy represents an attractive anti-cancer approach. Yet the effective therapeutic use of autophagy induction requires detailed knowledge of how the autophagy-lysosome pathway might be affected in cancer diseases. This is especially important given that disease-related genetic defects may affect autophagic pathway e.g., when lysosomal fusion or degradation is impaired. Thus, the stimulation of autophagy may rather worsen the disease progression. While autophagy modulation is an exciting area of clinical development, the effects of autophagy upregulation may vary substantially depending on the precise nature of the tumor state. Further comprehensive understanding of the roles of autophagic pathways throughout different stages of carcinogenesis has potential to guide development of novel therapeutic strategies to eradicate cancer cells with mutant p53. Furthermore, most if not all autophagy modulating drugs in clinical trials are inhibitors of the process, with the effectiveness of inhibiting autophagy to enhance chemotherapy cytotoxicity. Accordingly, pharmacological methods are not currently available to selectively and solely activate and target oncoproteins, including mutant p53, by autophagic pathways. While CMA can be directed to target oncogenic proteins, such as mutant p53, molecular mechanisms of its selective cargo recognition remain largely uncharacterized.

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

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REVIEW
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Mutant p53 Gain-of-Function: Role in Cancer Development, Progression, and Therapeutic Approaches

Eduardo Alvarado-Ortiz[1,2†], Karen Griselda de la Cruz-López[2,3†], Jared Becerril-Rico[2], Miguel Angel Sarabia-Sánchez[4], Elizabeth Ortiz-Sánchez[2] and Alejandro García-Carrancá[5]*

[1] Programa de Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Mexico City, Mexico, [2] Subdirección de Investigación Básica, Instituto Nacional de Cancerología, Secretaría de Salud, Mexico City, Mexico, [3] Doctorado en Ciencias Biomédicas, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico City, Mexico, [4] Programa de Posgrado en Ciencias Bioquímicas, Departamento de Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México, Mexico City, Mexico, [5] Laboratorio de Virus and Cáncer, Unidad de Investigación Biomédica en Cáncer, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México and Instituto Nacional de Cancerología, Secretaría de Salud, Mexico City, Mexico

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***Correspondence:**
Alejandro García-Carrancá
carranca@biomedicas.unam.mx

[†]These authors have contributed equally to this work

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Frequent p53 mutations (mutp53) not only abolish tumor suppressor capacities but confer various gain-of-function (GOF) activities that impacts molecules and pathways now regarded as central for tumor development and progression. Although the complete impact of GOF is still far from being fully understood, the effects on proliferation, migration, metabolic reprogramming, and immune evasion, among others, certainly constitute major driving forces for human tumors harboring them. In this review we discuss major molecular mechanisms driven by mutp53 GOF. We present novel mechanistic insights on their effects over key functional molecules and processes involved in cancer. We analyze new mechanistic insights impacting processes such as immune system evasion, metabolic reprogramming, and stemness. In particular, the increased lipogenic activity through the mevalonate pathway (MVA) and the alteration of metabolic homeostasis due to interactions between mutp53 and AMP-activated protein kinase (AMPK) and Sterol regulatory element-binding protein 1 (SREBP1) that impact anabolic pathways and favor metabolic reprograming. We address, in detail, the impact of mutp53 over metabolic reprogramming and the Warburg effect observed in cancer cells as a consequence, not only of loss-of-function of p53, but rather as an effect of GOF that is crucial for the imbalance between glycolysis and oxidative phosphorylation. Additionally, transcriptional activation of new targets, resulting from interaction of mutp53 with NF-kB, HIF-1α, or SREBP1, are presented and discussed. Finally, we discuss perspectives for targeting molecules and pathways involved in chemo-resistance of tumor cells resulting from mutp53 GOF. We discuss and stress the fact that the status of p53 currently constitutes one of the most relevant criteria to understand the role of autophagy as a survival mechanism in cancer, and propose new therapeutic approaches that could promote the reduction of GOF effects exercised by mutp53 in cancer.

Keywords: p53, gain of function, oncogenic pathways, metabolic reprogramming, stemness, chemo-resistance, immune evasion

INTRODUCTION

Cancer is a complex set of diseases, all characterized by abnormal cell growth, unresponsive to normal cellular and tissue controls. It originates with wayward cells that once formed, grow, expand, and ultimately disseminate to other parts of the body, and in many cases, when not detected early, will ultimately kill their host. Cancer cells are characterized by dysregulated key elements and fundamental signaling pathways controlling proliferation, cell-death, interactions with the immune system, metabolic changes, and response to drugs, among the most relevant (Hanahan and Weinberg, 2011).

The behavior and status of p53 is fundamental for cancer development, progression, and for the fate of many cancer patients. p53 plays many important roles in cancer and is considered a master regulator of intracellular functions, such that it has appeared on the covers of the most prominent science journals, like Science and, and has been awarded titles such as *"the guardian of the genome"* (Finlay et al., 1989; Soussi et al., 1990; Yeargin and Haas, 1995).

It is well established that altogether, around half of all human tumors exhibit alterations in *TP53* alleles, either by inactivation, loss or, importantly, mutations. Tumor cells containing mutant alleles of this gene generate mutant versions of the protein that, remarkably, mainly affect amino acids located within the DNA binding domain (DBD) (**Figure 1**). These mutant versions of p53 not only lead to loss of normal functions but surprisingly, confer mutant proteins with new abilities that provide cancer cells with key gain-of-function activities (GOF's).

Recently, the mechanisms and effects of these mutant alleles have been shown to affect key biological processes associated with cancer progression, invasion, metabolic reprograming, and interactions with the immune system. The study of such effects on central processes including proliferation, migration, generation of an inflammatory microenvironment, metabolic reprogramming, stem-cell restricted characteristics, and pharmacological resistance, has gained much attention. Although these processes are central for cancer, the molecular mechanisms involved and the precise targets acted upon by mutp53 GOF's, are only recently being elucidated.

Understanding the mechanisms involved and the effects of mutp53 GOF will be vital to better combat pharmacological resistance of cancer cells that harbor mutp53, and to design effective therapies based on p53 status in different types of cancer.

This review aims to integrate novel data on mechanisms and targets involved in the effects of mutp53 GOF's, stressing current knowledge of the central pathways involved.

DISCOVERY

The product of the *TP53* gene was first observed in the 1970's by several groups when studying cellular transformation of rodent cells induced by a simian virus called SV40. Transformation was observed when non-permissive cells were infected or rodents were injected with SV40, leading to tumor development and a strong host immune response against a viral protein called T antigen (TAg). Several groups used a monoclonal antibody to immunoprecipitate TAg from transformed cells. Although they observed a 53–54 kDa protein in polyacrylamide gels, the nature of this protein and its specific association with TAg was not evident (Chang et al., 1979). Simple experiments revealed this as a cellular protein specifically associated with TAg and two seminal papers suggested that this protein, named p53, represented a key element for viral transformation (Lane and Crawford, 1979; Linzer and Levine, 1979). A few years later, when a murine cDNA coding for TP53 was cloned and shown to transform fibroblasts in culture, it was stated that *TP53* was "just another oncogene" and was recognized as such for a long time (Oren and Levine, 1983; Parada et al., 1984).

Rearrangements of the *TP53* gene were found in several human tumors and more importantly, loss-of-heterozygosity, a characteristic of tumor suppressor genes, was commonly observed (Masuda et al., 1987). Although these different lines of evidence strongly suggested that *TP53* was not just another oncogene, at the time, few envisioned that it would emerge as prototype of all human tumor suppressor genes so far identified.

Finally, when the effect of that same human gene on transformed cells was studied, it clearly showed its nature as a tumor suppressor gene.

CANONICAL FUNCTIONS

A vast number of signals promote several p53-mediated functions, including cellular stress, DNA damage, hypoxia, nutritional stress, as well as differentiation signals. Activation of p53 (referred to as wtp53) drives a plethora of signals that fire different fundamental responses such as cell-cycle arrest, apoptosis, senescence, regulation of cellular energy metabolism, antioxidant defense, and immune system regulation (**Figure 2**). Relevant target genes of the p53 transcription factor encode proteins, such as p21 and p27 that induce cell-cycle arrest, PAI1 and CDKN1b involved in inducing senescence, PUMA, BAX, and NOXA involved in apoptosis, or TIGAR and GLS2 for metabolic changes, among others (Kastenhuber and Lowe, 2017; Simabuco et al., 2018).

One of best described roles for p53 is in the response to DNA damage. Acting as a classic transcription factor, p53 induces the expression of p21, which in turn inhibits CDKs (cyclin-dependent kinases), resulting in cell-cycle arrest (Deng et al., 1995).

Another function involves regulation of oxidative phosphorylation and mitochondrial respiration through the induction of COX (cyclooxygenase) and GLS2 (glutaminase 2) expression. GLS2 is an enzyme involved in deamination of glutamine, allowing production of α-ketoglutarate, an intermediary metabolite of the TCA cycle (tricarboxylic acid cycle) (Hu et al., 2010). Antioxidant defense mechanisms modulated by p53 include increasing levels of GST (glutathione S-transferase), an important enzyme implicated in avoiding the deleterious effects of ROS (reactive oxygen species) (Puzio-Kuter, 2011).

Furthermore, key functions of p53 include regulation of immune system interactions (Blagih et al., 2020). The main effector that allows p53 to orchestrate these interactions is NF-kB (Komarova et al., 2005). NF-kB constitutes a key transcription factor acting as one of the main regulators of pro-inflammatory activity in many immune responses. Its activation in leukocytes promotes the expression of pro-inflammatory cytokines (IL-1, IL-2, IL-6, TNF-α), chemokines (CXCL1, CXCL10, MCP-1), adhesion molecules (ICAM- 1, VCAM-1, ECAM-1), and anti-apoptotic factors (BCL-2, c-Flip, survivin) (Liu T. et al., 2017). NF-kB activation is normally suppressed by binding to its inhibitors, a family of proteins known as IkB.

Following a pro-inflammatory stimulus, IKKs (IkB kinases) are activated and phosphorylate IkB inhibitors, favoring their degradation, thus enabling NF-kB transcriptional activity (Dresselhaus and Meffert, 2019). NF-kB regulates recruitment, survival, proliferation, activation, and differentiation of leukocytes, in response to antigen recognition or activating signals (Liu T. et al., 2017). Different reports have confirmed the relationship between p53 and NF-kB, showing that wtp53 negatively controls the expression and activity of NF-kB (Gudkov et al., 2011; Blagih et al., 2020). This evidence indicates that regulation of wtp53 over the immune system is mainly due to a mutual repression between wtp53 and NF-kB that promotes an anti-inflammatory microenvironment.

However, wtp53 immune regulatory functions are not limited to repression of pro-inflammatory responses. For instance, MHC-I (Major Histocompatibility Complex Class I) is positively regulated by wtp53, promoting T cell recognition. Thus, alterations in *TP53* also lead to deficient immune system responses (Wang B. et al., 2013; Blagih et al., 2020).

Understanding wtp53 actions, it is possible to establish the effects derived from mutp53 GOF, being an important criterion in biological processes ranging from proliferation, migration, metabolism reprogramming, immune evasion, state of differentiation, and chemoresistance, which are explained in the following sections.

IMPACT OF MUTp53 GAIN-OF-FUNCTION

Although it is obvious that mutations in the *TP53* gene should result in loss of canonical functions, in the last years it has become evident that the most common mutant alleles acquire new functions that fuel tumor progression.

Most common GOF mutations in *TP53* are located within the DBD, as have been observed in many different types of human solid tumors. Conversely, p53 mutations located in the regulatory or tetramerization domains are less frequent. Common mutants have single missense mutations leading to single amino acid substitutions at key "hotspots" that preponderantly include residues 175, 248, and 273, as shown in **Figure 1A**. These high-frequency mutants represent non-random "hotspots" and correlate with poor cancer-free survival (**Figures 1B,C**).

There are two main types of mutant "hotspot" sites: contact mutants (R273H, R248Q, and R248W) and conformational mutants (R175H, G245S, R249S, and R282H), both affecting the DBD. Contact mutants generally produce structural changes in the p53 protein that directly affect DNA binding, while conformational mutants generate structural changes related with protein folding, both types of mutants have shown GOF activities (Kim and Lozano, 2018).

The effect of mutp53 is reflected on tumorigenic ability. Particularly, germline p53 mutants are mainly associated to Li-Fraumeni Syndrome, in which patients are more likely to develop tumors (Lang et al., 2004; Olive et al., 2004). Additionally, the effect of mutp53 has been described in transgenic mice able to express mutp53 in a tissue-specific manner (Wijnhoven et al., 2005). This versatility made it possible to determine that mutp53 can promote metastasis in a genetic context where mutant versions of other proteins, such as oncogenic Ras, are present (Morton et al., 2010). This evidence suggests that multiple oncogenic effects drive tumorigenesis and metastasis. In the case of colorectal cancer, even though loss of wtp53 improves the oncogenic capability of cancer cells through LOH, the presence of mutp53 in both alleles seems to be necessary to drive tumorigenesis (Nakayama et al., 2020). Moreover, intracellular accumulation of mutant versions of p53 increases the effects of signaling pathways affected by new GOF activities (Pfister and Prives, 2017).

Nuclear Effects of mutp53

A consensus response element (RE) is required for wtp53 to bind and regulate expression of target genes. However, the mutations in the p53 DBD lead to disruption in the ability to bind this RE. Importantly, mutp53 can bind to novel non-canonical DNA binding sites of several genes and thus, can positively or negatively regulate the expression of genes associated with malignancy (Kim and Deppert, 2007). This versatility of the nuclear effects of mutp53 has just recently been described, and it becomes relevant for the GOF associated with mutp53 (Göhler et al., 2005).

Even though DNA-binding of mutp53 does not depend on particular sequences, the mechanisms of mutp53-mediated transcription can be direct or indirect. Employing electromobility shift assays (EMSAS) and confocal fluorescence lifetime microscopy it was shown that mutp53 (248P and 245S) can bind specifically and selectively to non-B DNA. Binding does not require the presence of specific sequence motifs but indeed requires both the DBD and an intact p53 C-terminal regulatory domain (CRD). This mode of binding was termed as "DNA structure selective binding" (DSSB) (Göhler et al., 2005). In support of this, several mutant versions of p53 have been shown to bind preferentially to supercoiled DNA and by luciferase reporter assay demonstrated that the DNA topology influences p53 regulation of BAX and MSP/MST1 promoters (Brázdová et al., 2013). Moreover, it was shown that mutp53 binds efficiently to nonlinear DNA, and this can be increased by apurinic/apyrimidinic endonuclease 1/redox factor-1 (APE1) that stimulate DNA biding activity of numerous transcription factors in a redox-dependent manner (Cun et al., 2014).

For instance, mutp53 can bind to DNA on non-canonical sites from non-linear conformations (Göhler et al., 2005). It is important to mention that there are physical interactions

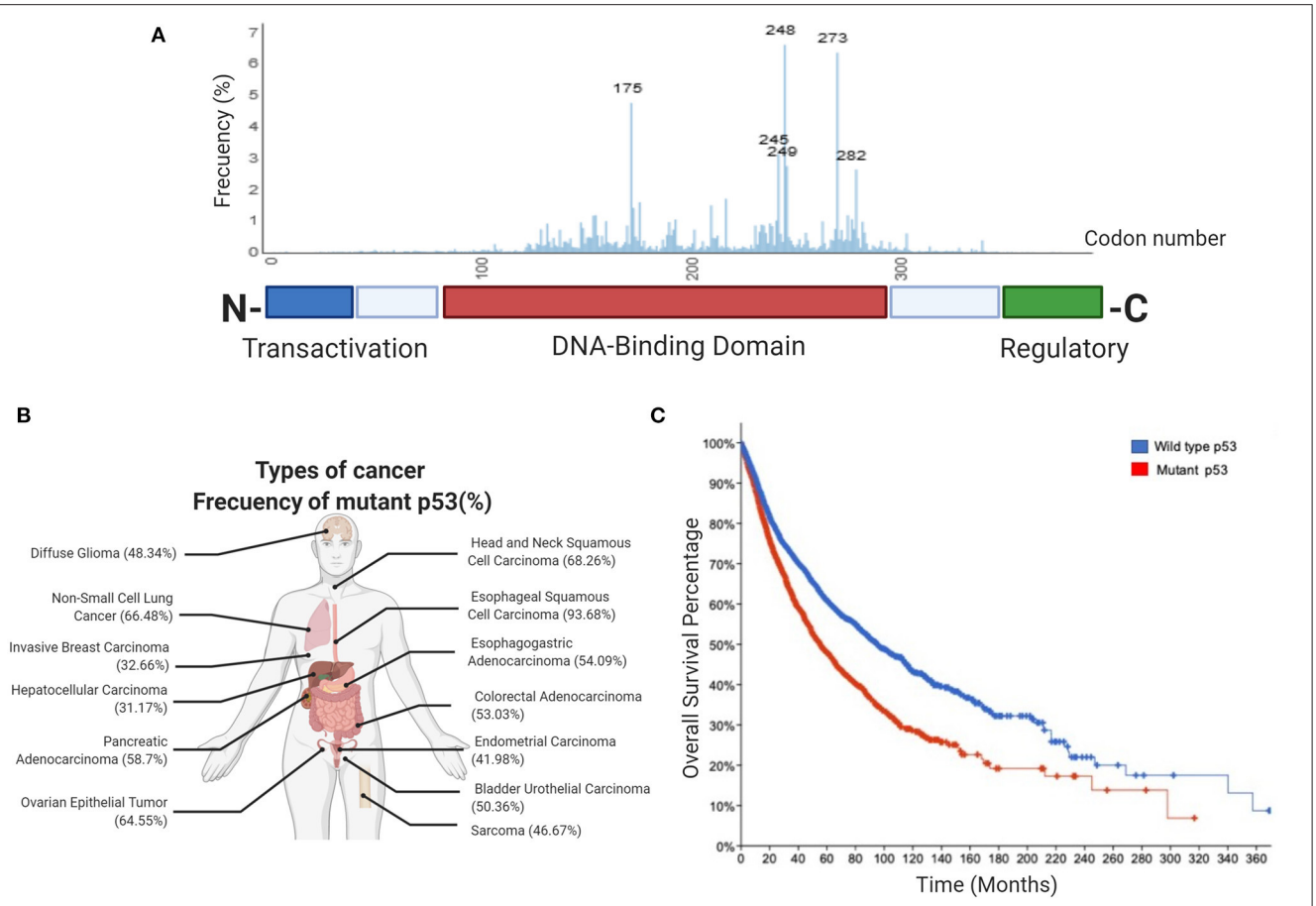

FIGURE 1 | Frequency of p53 mutations in human cancers. **(A)** Schematic picture showing the domain structure of the p53 protein, including the transactivation domain, DNA-binding domain and regulatory domain. The aligned graphs indicate the relative frequency of mutations across different domains of p53. p53 mutations are most frequently found in the DNA-binding domain, according to the IARC TP53 database. **(B)** Percentage frequency of TP53 gene alterations in different types of cancer. The data were obtained from TCGA PanCancer Atlas using a combined study (n = 10,967). **(C)** Overall survival for human cancer patients (N = 10,953 patients from 32 studies) with mutp53 (red line) or wild type p53 (blue line). The graph was analyzed and obtained from cBioportal.

of mutp53 with remodeling complexes that cause changes in the transcriptome, conferring plasticity in gene expression patterns. In this sense, it has been reported that binding of mutp53 to particular motifs on non-B DNA conformations, confers stability to mutp53, but also makes it more selective for modifying the activity of both transcription factors and chromatin remodelers (Göhler et al., 2005; Freed-Pastor and Prives, 2012). For instance, mutp53 has been related with the activity of the SWI/SNF chromatin complex, which increases histone modifications promoting an "open" state of chromatin, influencing the global transcriptome and the expression of cancer-related genes. The fact is that >40% of gene expression related to mutp53 can be explained by the effect on the SWI/SNF complex (Pfister et al., 2015).

Moreover, overlap in the DNA binding sequence patterns was observed through which mutp53 can act on response elements of other transcription factors, modulating gene expression (Agostino et al., 2006). For example, mutp53 can regulate gene expression through physical binding to p53 family members with tumor suppressor capacity such as p63 and p73, and modify their transcriptional activity (Ferraiuolo et al., 2016). Recently, it was shown that mutp53 interacts with the intracellular domain of Notch1 to abrogate p63/p73 mediated repression of HES1 and ECM, promoting lymphomagenesis (Zhang et al., 2019). A more complete picture has been shown with newer evidence pointing out that mutp53 can act as a nuclear repressive factor to downregulate pro-apoptotic responses, such as expression of the CD95 gene (Fas receptor), which is involved on apoptosis induction (Zalcenstein et al., 2003). Moreover, mutp53 is able to both positively and negatively regulate the activity of a variety of transcription factors, such as ETS2, NF-kB, HIF-1α, SMAD, SREBP, or NF-Y (Kim and Lozano, 2018). For instance, mutp53 may directly cooperate with YAP1 (Yes-associated protein) and favor the transcriptional activity of NF-Y on proliferation-related genes, suggesting that mutp53 may act as a transcription cofactor to enhance GOF (Di Agostino et al., 2016). The nuclear effect of mutp53 is an interesting field of study that is not well clarified.

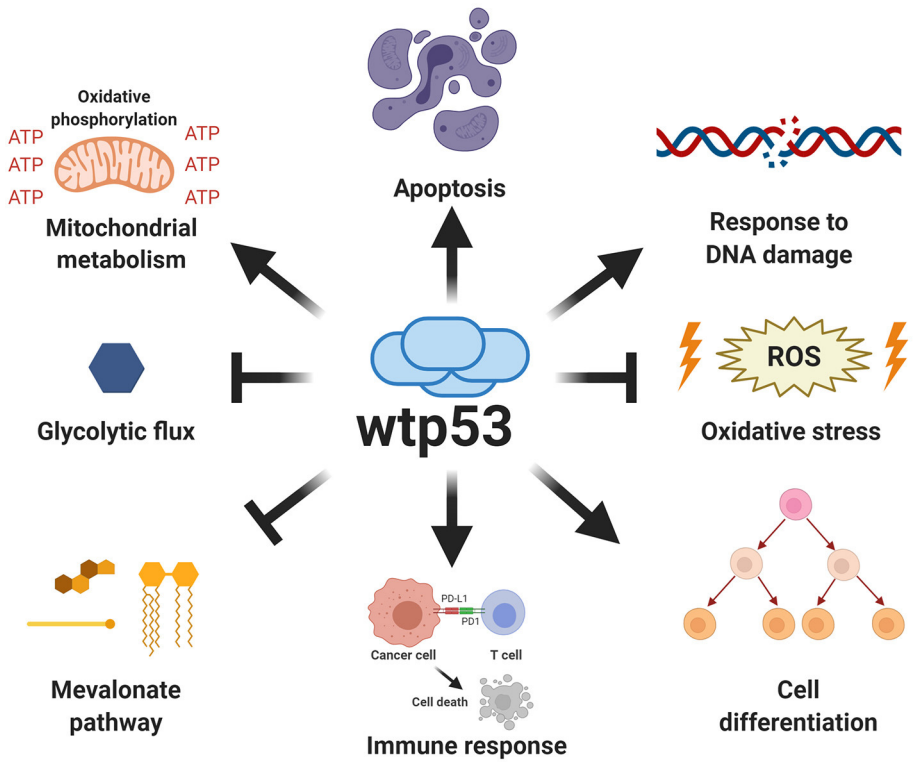

FIGURE 2 | Canonical functions of wild type p53. Wild type p53 is a major tumor suppressor whose functions are critical for protection against cancer. The canonical functions of wild type p53 include the induction of apoptosis, regulation of oxidative metabolism, and inhibition of glycolytic flux, as well as the response to DNA damage, increased antioxidant capabilities, regulation of immune response and differentiation processes.

Proliferation, Invasion and Metastasis

In the last decade, important contributions have allowed us to better understand the mechanisms involved and the impact of mutp53 GOF on cell proliferation, invasion and metastasis. The particular importance of mutp53 is in promoting proliferation, invasion and metastatic potential through its effect on the endosomal pathway, leading to recycling of receptors and integrins. For instance, overexpression of mutp53 has been shown to increase translocation of EGFR (Epidermal Growth Factor Receptor) and α5β1 integrin on the surface of cell membranes. This translocation is dependent on interaction with RCP (Rab-coupling protein) (Muller et al., 2009). As a consequence, many of the intracellular pathways associated with the regulation of endosomal pathways, including PI3K/AKT or MAPK cell signaling pathways, are activated by mutp53 (**Figure 3**).

Additionally, it has been reported that the R273H mutant binds and represses the promoter region of miR-27a, a microRNA that negatively regulates the EGFR transcript. This reinforces the evidence that the presence of mutp53 can favor the activity of signaling pathways related to EGFR, as well as the downstream signaling mechanisms. Gastric cancer tumor samples corroborate this effect, showing reduced expression of miR-27a compared to normal tissue (Wang W. et al., 2013).

Other effects attributed to mutp53 are mediated by the regulation of the PI3K/AKT pathway through binding DAB2IP (DAB2-interacting protein). DAB2IP is a scaffold protein that binds to and inactivates p85-PI3K, impairing its repressive functions over PI3K, promoting the intracellular effects of AKT1. Thus, growth factors, such as insulin, increase proliferation in prostate and breast cancer (Valentino et al., 2017).

Recent findings reveal that mutp53 (R175H) exacerbate the oncogenic response of K-Ras. The active state of K-Ras (G12C) is related to its GTP-binding form, while the GTPase-activating proteins (GAPs) can favor the GDP inactive form. Importantly, K-Ras activity is not enough to promote tumorigenic capacities in models such as pancreatic ductal adenocarcinoma, since the K-Ras mutant form cannot maintain the GTP-bound state. Nevertheless, mutp53 can regulate the splicing of GAPs through RNA-binding protein hnRNPK. The activity of mutp53 favors the expression of GAP isoforms that cannot bind to Ras, abrogating the ability to decrease its activity and supporting the oncogenic effect of K-Ras. This mechanism reveals a synergism between K-Ras and mutp53 through spliceosome effects, supporting malignant progression through the effect of multiple oncogenes like K-Ras (Escobar-Hoyos et al., 2020).

Additionally, it was found that mutp53 inhibits apoptosis associated with mitochondria and confers resistance to anoikis (Tan et al., 2015), a type of cell death related with loss of contact with the extracellular matrix or neighboring cells. Apoptosis associated with mitochondria requires the dissociation of the proapoptotic protein BIM from the antiapoptotic

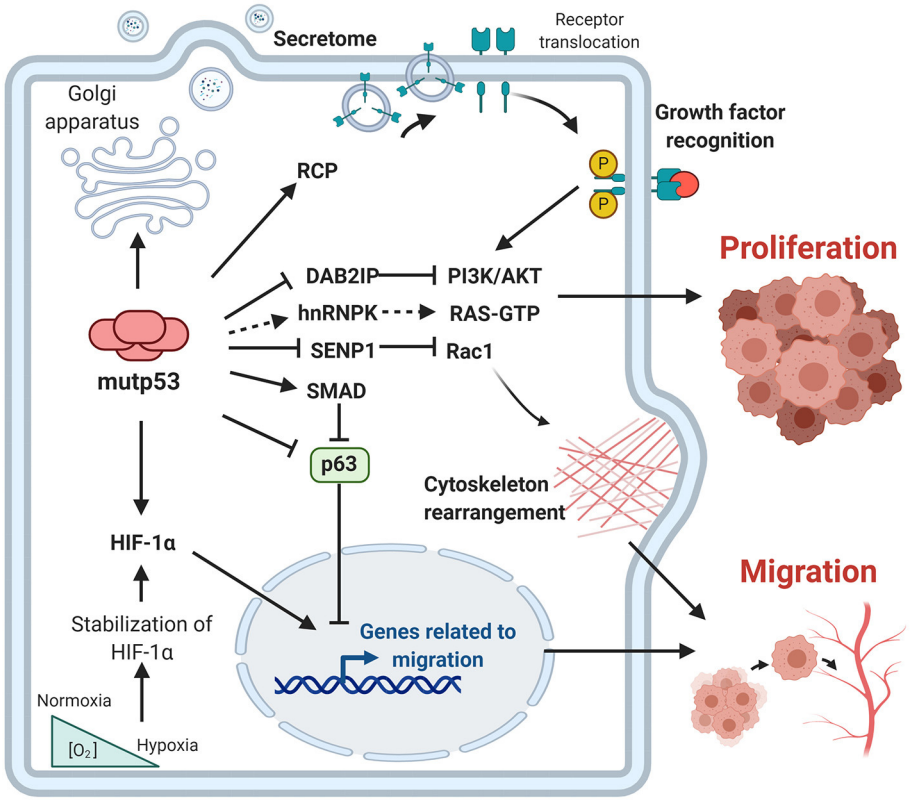

FIGURE 3 | Gain-of-function of mutant p53 over proliferation, invasion and metastasis. The principal GOF activities of mutant p53 have nuclear and non-nuclear effects. The nuclear effects involve binding to transcription factors such as HIF-1α or p63 and p73, while the non-nuclear effects are regulation of intracellular proteins, such as RCP, implicated in receptor translocation, DAB2IP scaffold protein implicated in the PI3K/AKT pathway, or SENP1, a protease related to Rac1 activity.

protein BCL-XL. However, the presence of the p53-R273H mutant suppresses BMF (BCL2-modifying factor) expression, which is a protein that induces cellular anoikis and apoptosis by reducing the interaction between BIM and BCL-XL. Knockdown of endogenous mutp53 restores sensitivity to apoptosis, highlighting the importance of mutp53 not only in cellular proliferation, but in cell survival of lung, colon, and breast cancer cell lines (Tan et al., 2015).

Considering receptor recycling generated by RCP, it has been suggested that cellular scattering could be attributed to HGF (Hepatocyte growth factor) as well as the presence of mutp53. Both elements potentiate MET (HGF receptor) activity, increasing its phosphorylation. The main biological responses are cytoskeletal changes that allow cell motility (Jo et al., 2000). The presence of mutp53 not only exacerbates migratory abilities through the MET receptor, but also promotes inhibition of p63, a key transcription factor regulating expression of anti-metastatic genes, evidencing that the effects of mutp53 are not limited to a particular mechanism (Muller et al., 2009, 2013).

Moreover, in a model of endometrial cancer mutp53 can promote EMT (epithelial-mesenchymal transition). Studies on miR130b, have determined that mutp53 is partially responsible for promoting an invasive phenotype through binding of mutp53 to the promoter region of this microRNA, thereby repressing its transcription. MiR130b inhibits Zeb1 expression, a transcription factor involved in regulating the expression of EMT markers. Thus, mutp53 represses transcription of miR130b and increases transcription of Zeb1, favoring invasion (Dong et al., 2013).

Studies employing immunoprecipitation assays have shown that mutp53 interacts with the small GTPase Rac1 and inhibits its interaction with SUMO-specific protease 1 (SENP1) favoring an active state of Rac1 (Yue et al., 2017). Thus, Rac1 activation is an important mechanism by which mutant p53 GOF promotes tumor metastasis.

Furthermore, accumulation of versions of mutant p53 seems to favor GOF and the chaperone machinery mediated by Hsp90 partially explains mutp53 stabilization. Furthermore, Hsp90 can be secreted by cancer cells, specifically those with mutp53 (R175H), influencing ECM degradation as well as migratory capacities. This effect is explained by the mutp53/RCP axis, favoring colonization to distant sites, such as the lung. Importantly, targeting the extracellular effect of Hsp90 decreases the invasive capacities of mutp53 cancer cells (Zhang S. et al., 2020). This evidence opens new avenues for the use of Hsp90 inhibitors in patients with mutp53.

Part of the effects of mutp53 over migration rely on other members of the p53 family, which include p63 and p73 transcription factors. These transcription factors share a

conserved DBD that allows them to regulate the expression of a common pool of genes that are crucial for preventing tumorigenesis. Although p63 and p73 form homo and hetero tetramers, neither can bind to wtp53. Conversely, it has been found that several mutp53 versions can interact with both p63 and p73, and inhibit their transcriptional activity. It was shown that the recombinant core domain of some mutp53 proteins, but not wtp53, binds and inhibits p63 by masking its DBD (Gaiddon et al., 2001; Strano et al., 2001).

It is well accepted that GOF of mutp53 includes the ability to sequester the transactivation (TA) domain isoform of p63 and inhibit its interaction with its canonical DNA response element, thereby disrupting its downstream anti-metastatic transcriptional networks (Strano et al., 2001). Additionally, Neilsen et al. (2011) demonstrated that mutp53 GOF activities aberrantly alter the gene expression pattern of cancer cells to promote oncogenesis, involving a collaborative approach with p63 transcription factor. They show that mutp53 uses p63 as a molecular chaperone to bind to the promoter of target genes causing reprograming of the transcriptome. These genes are mainly associated with cellular invasion. These studies show that mutp53 can induce the secretion of pro-invasive factors to the surrounding microenvironment (Neilsen et al., 2011).

Importantly, the effect of mutp53 over p63 is decisive for signaling pathways like TGF-β (Transforming Growth Factor β), to determine whether they act as tumor suppressors or promoters of cellular migration and metastasis. Extracellular TGF-β receptor ligands exert their actions through Smad 2/3 transcription factors. Under non-cancerous contexts, they act as cell growth suppressors, but in the presence of mutp53 they improve migration ability, highlighting the pleiotropic relevance of TGF-β in cancer. Interestingly, it was shown that p63 is functionally inactivated when complexed with mutp53 and Smad in the presence of TGF-β ligands, this being critical for supporting metastasis. Moreover, this process is dependent on mutp53 N-terminal phosphorylation by oncogenic Ras. Mechanistically, mutp53 and Smad intercept p63 to form a ternary complex in which the p63 transcriptional functions are antagonized, offering an interesting explanation for the migratory effects induced by TGF-β (Adorno et al., 2009). Additionally, other reports revealed that mutp53 binds to the MH2 domain of Smad3, promoting a decrease in canonical TGF-β pathway signaling (Ji et al., 2015).

Moreover, studies have shown that part of the functions of mutp53 are involved with adapting to a hypoxic microenvironment, which favors an invasive phenotype. In this sense, there is dual participation between mutp53/HIF-1α, which allows for increased expression of extracellular matrix proteins, such as VIIa1 collagen and laminin-γ2, promoting an invasive phenotype in non-small cell lung cancer (Kamat et al., 2007; Amelio et al., 2018). Other reports support this premise, since it has been shown that there is an increase in tumor vascularization, as reflected by VEGF expression, as well as an increase of ROS in cell lines with mutp53 status (Khromova et al., 2009).

This is reinforced by evidence suggesting that the expression of pro-angiogenic isoforms of VEGF, but not anti-angiogenic isoforms, seem to depend on the interaction between mutp53 and the ribonucleoprotein complex composed by MALAT1 lncRNA, SRSF1, and ID4, favoring splicing of VEGF pro-angiogenic isoforms. This being an important axis in breast cancer cells (Pruszko et al., 2017). Recently, it has been reported that the effect of mutp53 reflects on morphological alterations of the Golgi apparatus which lead to alteration of the secretome of cancer cells, promoting release of soluble factors into tumoral microenvironment, including VEGF. From a mechanistic overview, this effect is explained by the dual action of mutp53 and HIF-1α through miR-30d, under both hypoxia and normoxia conditions. The secretome alteration promoted by mutp53 exercises important effects on primary and distant sites during carcinogenesis (Capaci et al., 2020).

Metabolic Reprogramming

Hyperactivation of oncogenic pathways directly regulates the metabolic pathways that support tumor growth. Interestingly, mutp53 has been shown to enhance the Warburg effect, a process characterized by an increase in glucose uptake and lactate secretion even in the presence of oxygen (Levine and Puzio-Kuter, 2010; Eriksson et al., 2017). It was shown that mutp53 increases translocation of the glucose transporter GLUT1, without affecting total protein levels, favoring glucose uptake. The mechanistic effect is explained by an upregulation of the RhoA pathway. RhoA is a protein involved in different intracellular pathways like the activation of the effectors, ROCK1/2, which has been demonstrated to improve the distribution of transporters to the cell membrane in different cell types. Impairment at different points of the mutp53/RhoA/ROCK axis promotes an important decrease in glycolytic flux in different types of cancer cell lines (Zhang et al., 2013). This constitutes one of the first reports that explains how the presence of mutp53 favors the Warburg effect. Moreover, other reports support this evidence, showing that wtp53 antagonizes the Warburg effect and favors oxidative phosphorylation (Zhou et al., 2014; Hernández-Reséndiz et al., 2015).

Some authors have recently focused on the regulation of the mevalonate (MVA) pathway implicated in lipid metabolism and posttranslational modifications related to the malignant process. The biological effects of mutp53 on the regulation of the MVA pathway explain various cellular processes ranging from proliferation to fitness, or the regulation of the tumor microenvironment, all of them with functional relevance for tumorigenesis (Mullen et al., 2016; Ingallina et al., 2018).

Generation of MVA requires sequential action of enzymes, among which HMGCR constitutes a key element. Transcriptional regulation of HMGCR is controlled by SREBP (Sterol Regulatory Element-Binding Protein), which recognizes sterol-response elements on its promoter region (Mullen et al., 2016). Importantly, simultaneous binding of mutp53 and SREBP has been demonstrated on promoter regions recognized by SREBP using ChIP assays of genes implicated in the MVA pathway, including HMGCR, in breast cancer (Freed-Pastor et al., 2012). Thus, a great number of small GTPases, such as Rho and Ras, whose post-translational

modifications are regulated downstream of MVA pathway can be increased by mutp53 (Freed-Pastor et al., 2012; Parrales et al., 2016).

The increased activation of anabolic pathways is an essential characteristic of cancer cells because they enable production of the macromolecules required for replicative cell division and tumor growth. One of the proposed mechanisms through which mutp53 favors the activation of anabolic pathways relies on AMPK inhibition, contrary to wtp53, which increases AMPK activity (Feng et al., 2007). AMPK is a Ser/Thr kinase activated by an increase in AMP levels, caused by energy stress (Zhou et al., 2014). AMPK decreases anabolic pathways such as fatty acid synthesis and protein synthesis, and promotes catabolic pathways including oxidation of fatty acids and autophagy. Mutp53 (R175H) can bind directly to the AMPKα subunit, thereby inhibiting activation of AMPK by upstream kinases. The consequences of this interaction, besides AMPK inhibition, is that the downstream targets of this kinase are not being regulated, and therefore, there is an increase in glycolytic flux, as shown in **Figure 4** (Zhou et al., 2014).

Metabolic alteration and GOF related to mutp53 in cancer cells can increase the levels of reactive oxygen species (ROS). Moreover, the presence of mutp53 decreases NRF2 (Nuclear factor erythroid 2–related factor 2) activity and glutathione synthesis, promoting ROS accumulation (Liu D. S. et al., 2017). Conversely, it has been widely demonstrated that wtp53 has important role in regulating ROS levels and therefore, in determining the stress response. One mechanism is through the regulation of TIGAR (TP53-induced glycolysis and apoptosis regulator), a transcriptional target of wtp53. TIGAR shares sequence similarities with the bisphosphatase domain (FBPase-2) of the bifunctional enzyme PFK-2/FBPase-2 (6-phosphofructo-2kinase/fructose-2,6-bisphosphatase). These well-known functions lead to glycolysis blockage and favor the production of NADPH through pentose phosphate. This mechanism represents an important mechanism for wtp53 to favor antioxidant capacities, promoting ROS scavenging (Bensaad et al., 2006) Thus, it is not surprising that presence of mutp53 drives an imbalance between glycolysis and oxidative phosphorylation, as well an increase in oxidative stress. Importantly, TIGAR expression has been reported under conditions where mutant versions of p53 are present. Under these conditions, TIGAR plays a key role in protecting cancer cells from oxidative stress generated by sustained proliferation (Cheung et al., 2013). This evidence supports a dynamism between the functions of p53 to adapt to survival under stress conditions.

Recently, it has been shown that cancer cells can adapt to stress conditions. Availability of glutamine in the tumor microenvironment allows cancer cells with mutp53 to generate adaptive mechanisms to avoid apoptosis. However, although glutamine constitutes an important energy fuel for proliferation, cancer cells with mutp53 (R288, R280) can adapt to stress conditions, even in the absence of glutamine. In accordance with this, it was shown that mutp53 can reestablish canonical p53 transcriptional activity over a particular set of genes, such as GLS1, CDKN1A, GAGG45A, and TIGAR, favoring new adaptive mechanisms for stress conditions (Tran et al., 2017; Ishak Gabra et al., 2018).

Immune System Regulation

Genetic alterations of cancer cells induced by the malignant transformation process has an important effect in the ability to be recognized by the immune system. During recent years, p53 has emerged as one of the major regulators of cancer-immune system interactions (Blagih et al., 2020). The dynamic and bidirectional relationship between tumor cells and the microenvironment has been evidenced to be decisive for the establishment and progression of tumors (Wang et al., 2017). One of the main microenvironment components that allows the development of tumor growth is the immune system; this relationship was already being contemplated in the nineteenth century as a predisposing factor for cancer disease (Gonzalez et al., 2018). It is well accepted that under normal conditions, the immune system seeks to eliminate cells with aberrant characteristics, however, modifications in the functions of neoplastic cells not only prevent elimination, but even benefit from the inflammatory functions of the immune system (**Figure 5**).

Tumor progression is generally associated with immune system evasion, and loss-of-canonical function of wtp53 stands as a crucial point for the generation of an inflammatory microenvironment that not only limits the immune system response but indeed, benefits cancer cells (Blagih et al., 2020).

As previously discussed, wtp53 acts as a repressor of pro-inflammatory activity through the inhibition of NF-kB, which in turn is also able to inhibit wtp53 activity, thus promoting cell survival and proliferation. This repressive function of wtp53 over NF-kB is impaired by the presence of p53 mutants, acting in an opposite manner, due to the stimulatory effect of mutp53 on NF-kB activity after exposure to TNF-α (Webster and Perkins, 1999; Weisz et al., 2007). The pro-inflammatory and pro-tumorigenic effect of TNF-α, orchestrated by mutp53, results from the interaction between mutp53 and NF-kB. Interestingly, when both factors are bound on promoter regions of cancer-related genes, such as MMP9 and CCL2, they favor an active chromatin, improving transcriptional activity (Cooks et al., 2013; Rahnamoun et al., 2017). The interaction between mut53 and NF-kB is persistent over time, generating inflammation-driven colon cancer (Cooks et al., 2013; Uehara and Tanaka, 2018).

Thus, it is evident that mutp53 tumor cells have greater tumorigenic and migratory capabilities after a pro-inflammatory stimulus, as well as an increase in pro-inflammatory cytokine expression. In addition to TNF-α, IL-8 is also increased in mutp53 cancer cell lines. It is known that IL-8 is a pro-inflammatory cytokine that shows a high capacity for chemotaxis toward neutrophils and whose expression is found to be dependent on NF-kB (Hidaka et al., 2005; David et al., 2016). Consequences of IL-8 over-expression include an increase in tumorigenic properties, the EMT process, as well as improving stemness of tumor cells (Long et al., 2016). Additionally, it has been observed that tumor cells carrying the endogenous R273H mutp53 suppress the activity of sIL-1Ra, an antagonist of the IL-1R (interleukin 1 receptor), through an interaction between mutp53 and the transcription factor MAFF (MAF bZIP

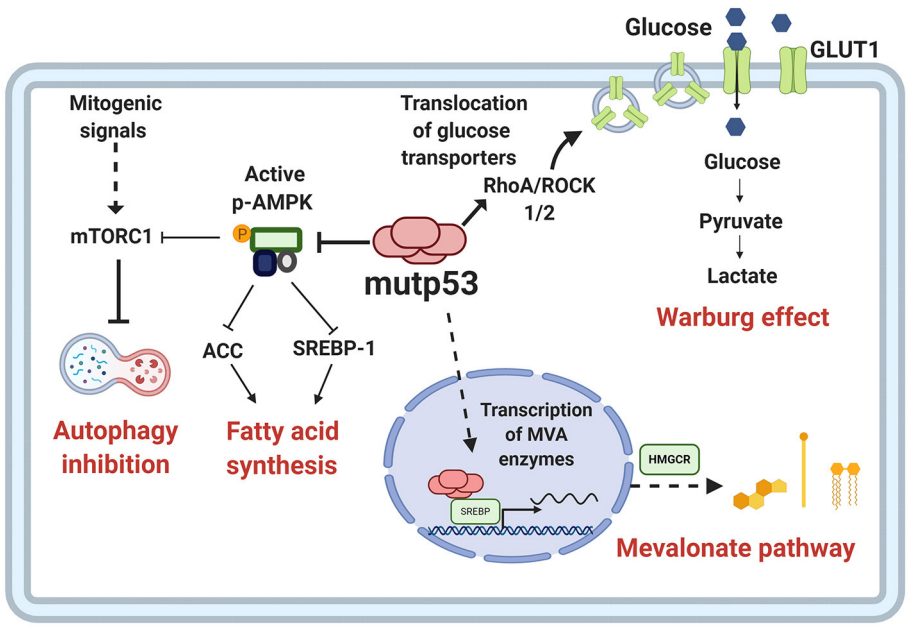

FIGURE 4 | Metabolic reprogramming by mutp53. Mutp53 GOF activities are involved in different critical points of tumor metabolism. Mutp53 favors glucose uptake and hence the Warburg effect through membrane translocation of the glucose transporter, GLUT1, via the RhoA/ROCK1/2 axis. Moreover, mutp53 can induce the Warburg effect by directly inhibiting AMP-activated protein kinase (AMPK), a major cellular energy sensor and a master regulator of metabolic homeostasis. AMPK downregulates fatty acid synthesis by inhibiting transcription factor sterol regulatory element-binding protein 1 (SREBP1). Mutp53 increases the activity of SREBP1, a master regulator of fatty acids and cholesterol biosynthesis, and thus, the mevalonate (MVA) pathway.

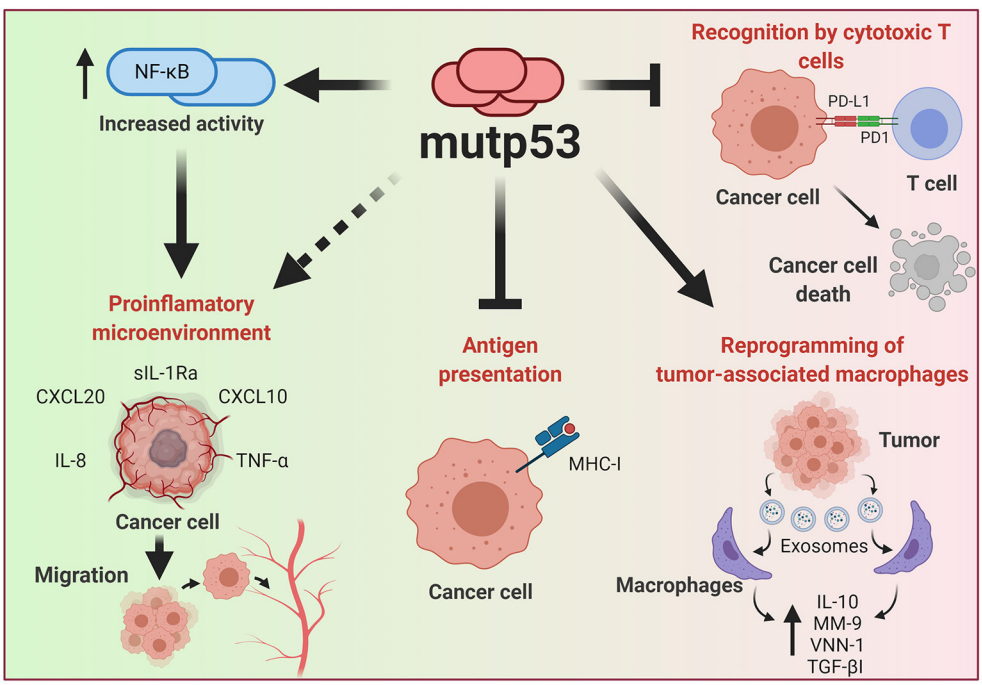

FIGURE 5 | Effect of mutp53 over the immune system. Mutp53 supports a pro-inflammatory microenvironment through the release of siL-1RA, CXCL20, CXXCL10, IL-8, or TNF-α, mainly by increasing the transcriptional activity of NF-kB. Additionally, the presence of mutp53 decreases MHC-I expression, avoiding recognition by T cells. Tumor cells can liberate exosomes that act over neighboring macrophages and improve IL-10, MM-9, VNN-1, and TGF-βI release, thus creating a microenvironment that improves cancer progression. Moreover, mutp53 can increase PD-L1, constituting an important mechanism for avoiding the oncolytic activity of T cells.

Transcription factor F), inhibiting its activity. In this manner, the interaction of the R273H mutant with the MAFF transcription factor, prevents the suppressive action of sIL-Ra on IL-1R, amplifying the pro-inflammatory and tumorigenic activities of IL-1 in colon and breast cancer cell lines (Kannan et al., 2012; Ubertini et al., 2015).

Among the pro-inflammatory cytokines expressed in tumor cells with mutp53 are those which recruit leukocytes, such as macrophages, neutrophils, dendritic cells, and lymphocytes. For example, overexpression of CXCL10, CX3CL1, and LTB chemokines in breast cancer, generates chemotaxis of T lymphocytes, cytotoxic T lymphocytes, as well as NK (Natural Killer) cells, through a mechanism dependent on DAB2IP protein inhibition, thus promoting pro-inflammatory and cell migration activities (Di Minin et al., 2014). This is derived from the DAB2IP repressive activity on pro-inflammatory signaling pathways such as NF-kB, and as previously mentioned, on tumorigenic pathways such as the PI3K/AKT cell signaling pathway. Therefore, the inhibition of DAB2IP through its protein-protein interaction with mutp53 (R176H, R280K), promotes the activation of these pathways (Bellazzo et al., 2017; Valentino et al., 2017). Additionally, in breast cancer cell lines, overexpression of the CXCR4 receptor (whose ligand is the CXCL12 chemokine) has been found in mutp53 cells, improving their migratory capabilities (Mehta et al., 2007).

It has recently been found that this GOF for cytokine release can be accomplished through exosome-mediated mechanisms. Under co-culture conditions of colon cancer cell lines (expressing endogenous mutp53) with M0 and M2 macrophages, it has been observed that the macrophages showed an increase in the release of IL-10, MM-9 (metallopeptidase matrix 9), VNN-1 (non-inflammatory vascular molecule 1), and TGF-βI, due to the action of miR-1246-containing exosomes secreted by tumor cells. This was corroborated in colon tissue samples from patients with mutp53 (Cooks et al., 2018). This anti-inflammatory microenvironment causes a failure in tumor cell elimination by the immune system and, additionally generates activities that favor cellular metastasis due to the destruction of the extracellular matrix and the formation of new blood vessels.

This establishes that mutp53, not only generates a pro-inflammatory environment but also anti-inflammatory ones. Other studies show an over-expression of immunological checkpoints that facilitate immune system evasion by mutp53 tumor cells. In breast cancer patients, over-expression of molecules associated with anti-inflammatory environments such as CTL4, PD-L1, PD-L2, PD-1, LAG2, BTLA, and TIGIT was confirmed in tumors with mutp53, being associated with the prognosis of the disease (Liu et al., 2019).

Similarly, over-expression of the transmembrane protein PD-L1 (Programmed Death-Ligand 1) has been found in mutp53 lung cancer and melanoma cells (Cortez et al., 2016; Thiem et al., 2019). Its main function is the suppression of the pro-inflammatory activity of T cells after the recognition of their specific antigen by interaction of TCR (T cell receptor) with MHC (major histocompatibility complex), being a regulatory mechanism of the inflammatory response (Akinleye and Rasool, 2019). Additionally, the activation of the JAK-STAT pathway by INF-γ receptors generates PD-L1 overexpression (Akinleye and Rasool, 2019). However, in neoplastic cells, mutp53 has been shown to generate low levels of miR-34a, which enables PD-L1 overexpression (Cortez et al., 2016). Additionally, in melanoma, the presence of mutp53 also leads to PD-L1 overexpression and a lower activity of cytotoxic T-cells over tumor cells (Thiem et al., 2019).

Conversely, the positive regulation of wtp53 over MHC-I establishes a relationship between p53 and oncolytic activity by T cells. Taking into account that mutp53 cells show low levels of MHC-I, this could represent an important barrier for T cell recognition. Recently, it has been proposed that low doses of TNF (Tumor Necrosis Factor) can rescue the expression of MHC, making mutp53 cancer cells more sensitive to immunological therapy (Garancher et al., 2020).

This dual role of mutant p53 versions to induce both pro-inflammatory and anti-inflammatory environments becomes a challenge for the immunological eradication of cancer. It is possible that this process is related to the different tumorigenic stages of cancer, and therefore with different microenvironment requirements for tumor progression or favoring certain cellular subsets (Gonzalez et al., 2018).

Conferring Stemness

It is now accepted that the majority of tumors exhibit a hierarchy of cells within the tumor, where stem-like cells are positioned at the top and are referred to as CSC (Cancer Stem Cells). Under physiological conditions, tissues are subject to constant renewal, and decisions between self-renewal of tissue stem-cells or cell differentiation are associated with wtp53 activity (Solozobova, 2011).

The advancement of genetically modified models provides information about the relevance of the relationship between p53 function and maintenance of the stem cell pool that might provide precursors for tumor initiation. For instance, transgenic mice harboring mutp53 developed malignant glioma, mainly detecting cells in the corpus callosum and olfactory bulb, both migratory destinations for stem cells residing in the subventricular zone. This suggests that neural stem cells or progenitors are mediating gliomagenesis caused by mutp53 (Wang et al., 2009).

Another example was observed in hematopoietic stem cells, where the R172H mutp53 promoted greater ability to self-renew *in vitro* and *in vivo* compared to wtp53 loss, showing that FOXH1, a regulator of stem cell factor receptor c-Kit and SCA-1 (Stem Cell Antigen 1), was necessary for this phenotype in cells expressing mutp53 (Loizou et al., 2019).

Following this notion, mice harboring the R248Q mutp53 favor tumor development, compared to mice with other mutations, such as G245S, since R248Q alters the stem cell compartments, by improving survival and self-renewal of hematopoietic and mesenchymal stem cells, putative primary malignant cells. This explains the similarity with Li-Fraumeni patients, a familial cancer predisposition, in which the R248Q mutp53 increases tumor initiation compared to other mutants, possibly because the R248Q mutp53 is able to co-aggregate

into higher-order structures with other tumor-suppressor transcription factors (Xu et al., 2011; Hanel et al., 2013).

Some types of cancer are originated through age-related mutations. This can be evidenced in C57BL/6, a type of old mice vulnerable to developing fibrosarcoma, where it was demonstrated that mesenchymal stem cells isolated and cultured *in vitro* were spontaneously transformed. The acquisition of tumorigenic potential was accompanied by the expression of stemness factors such as Klf4, Oct4, Sox2, c-Myc, as well as by the expression of mutp53 (Li et al., 2007).

The self-renewal capacity of undifferentiated populations requires a balance between "open" and "closed" state of the chromatin. During the stemness of embryonic cells, a bivalent state has been identified that reflects posttranslational modifications of histones that can generate a transcriptionally inactive state. PRC1 and PRC2 (Polycomb Repressive Complex 1 and 2) act in an orchestrated way to keep this repressive state, defining specific lineages. However, in the case of cancer, these mechanisms can regulate oncogenic functions through silencing of tumor suppressor genes (Laugesen et al., 2016). Interestingly, the presence of wtp53 seems to be determinant in controlling these epigenetic modifications.

Recently, it has been found that mutp53 triggers self-renewal of hematopoietic stem cells by increasing levels of H3K27me3 and therefore promoting a repressive chromatin state. In this study, three mutp53 versions (R248W, R273H and R175H) showed increased association with EZH2 (Enhancer of Zeste Homolog 2), which is part of PCR2 (Polycomb Repressive Complex-2), compared with wtp53, improving EZH2 binding to chromatin. Nonetheless, mutp53 promoted the presence of H3K27me3 rather than altering genomic distribution (Chen et al., 2019). Moreover, mutp53 also indirectly upregulates EZH2 by attenuating miR-26a, a negative regulator of EZH2, supporting another mechanism in the regulation of EZH2 activity (Jiang et al., 2015).

One of the main challenges in the study of CSC has been the development of appropriate tools that allow distinguishing them from the rest of the cancer cells. Surface protein markers have allowed addressing this problem, predominantly employing CD44, LGR5, and CD133 (Barker et al., 2007; Keysar and Jimeno, 2010; Alvarado-Ortiz et al., 2019). It was shown that wtp53 inhibits CD44 expression in breast cancer cells, but R248H mutp53 increased $CD44^+$ cells in colorectal cancer (Zeilstra et al., 2013; Solomon et al., 2018).

Solomon and collaborators showed the relationship between p53 functionality and CSC properties in colorectal cancer. They showed that in the RKO cell line, which endogenously expresses wtp53 but was transfected with R248H mutp53, there was an increase in the number of $LGR5^+$ and $CD44^+$ cells. Conversely, knockdown of endogenous mutp53 in SW480 cells diminished $CD44^+$ cells (Solomon et al., 2018).

In addition to surface proteins, CSC can also be identified through high enzymatic activity of proteins such as ALDH (Aldehyde Dehydrogenase) ($ALDH^{HIGH}$ cells) (Toledo-Guzmán et al., 2019). The $ALDH^{HIGH}$ population was augmented in cells that overexpress mutp53, while it was reduced by mutp53 knockdown (Solomon et al., 2018; Zhao et al., 2019). Furthermore, ALDH levels were upregulated in colorectal tumor samples expressing p53 missense mutations and clinically associated to higher aggressiveness. This poor prognosis seems to be linked to CSC-related capabilities, such as higher tumorigenic potential and chemo-resistance, which agrees with the proposal that mutp53 favors chemo-resistance and its absence leads to chemo-sensitivity (**Figure 6**).

Interestingly, mutp53 binds to promoter sequences of ALDH1A1, CD44, and LGR5. ALDH1A1 being implicated in mutp53-mediated chemo-resistance (Chen et al., 2016; Solomon et al., 2018). ALDH1A1 belongs to the ALDH family of enzymes and is capable of metabolizing, not only endogenous substrates but also inactivates some drugs used in chemotherapy, especially aldophosphamides. Remarkably, Gui et al. (2020) found that while the presence of wtp53 preferentially associates with a dominant ALDH isoform in tumors from HNSCC (Head and Neck Squamous Cell Carcinomas), mutp53 displayed a different diversity of ALDH isoforms, thus severely influencing chemoresistance associated to ALDH.

Chemo-resistance involves capabilities such as efflux of exogenous agents. Therefore, artificial dyes are used to identify these cells *in vitro* and CSC are then referred to as SP (Side Population). Analysis of SP in colorectal cancer-derived cell lines show that DLD-1 cells expressing mutp53 and Caco-2 (p53 null) cells showed a SP, while in HCT116 cells harboring wtp53, it was hardly detected. This is in agreement with the evidence that wtp53 inhibits MDR genes. Therefore, alterations in the p53 gene would impact the drug efflux capacity of the cells (Allen et al., 2009).

Functionally, CSC also are capable of serially forming spheres *in vitro* and display higher tumorigenic potential under *in vivo* conditions. An example was confirmed by Zhao et al. (2019) where overexpression of R273H mutp53 in a p53-null cell line showed elevated sphere formation and an increased expression of Sox2 and Nanog, favoring greater tumor initiating capacity. Furthermore, normal astrocytes expressing mutp53 were able to form spheres, emphasizing the potential of mutp53 in non-stem cells to trigger a CSC-like state (Escoll et al., 2017).

Supporting evidence for molecular mechanisms that allow self-renewal in the presence of mutp53 are scarce. The canonical Wnt pathway represents the most likely candidate, since it is commonly associated with self-renewal of stem cells, and its activity seems to be dependent on p53 status (Nusse and Clevers, 2017). The β-catenin protein is the transcriptional cofactor involved in Wnt signal transduction, and is modified post-translationally to regulate its functions.

It is well known that wtp53 acts negatively in regulating the canonical Wnt pathway (Kim et al., 2011). It is noteworthy that mutp53 increases β-catenin levels (Cagatay and Ozturk, 2002), possibly through Siah1 (Seven in absentia homolog 1) regulation, a target of wtp53 that participates in β-catenin degradation (Fiucci et al., 2004; Xie et al., 2009). Another mechanism related to β-catenin levels is mediated by mir-34a, a p53 transcriptional target, which downregulates β-catenin mRNA. In this regard, it is striking that mir-34a targets additional components of the Wnt pathway, decreasing β-catenin-dependent transcription, including stemness-related genes (Kim et al., 2011).

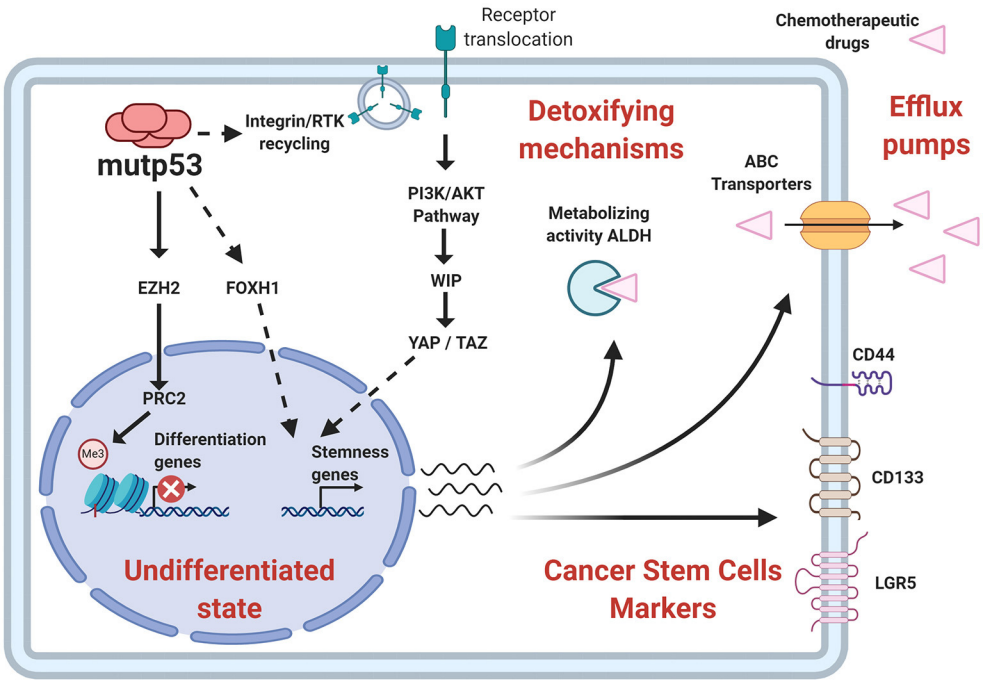

FIGURE 6 | Relationship of stemness properties with mutp53. Mechanisms related to self-renewal pathways favored by mutp53 include an increased EZH2 (subunit of PCR2 complex) activity and improvement of epigenetic modifications associated with a repressive state of chromatin. Other pathways include YAP/TAZ activity, as well as nuclear effects of FOXH1. Additionally, mutp53 increases CSC markers, such as CD44, CD133, LGR5, and the enzymatic activity of ALDH, contributing to stemness and pharmacological resistance.

Therefore, the inhibitory role of wtp53 over the Wnt pathway is consistent with an increase in transcription mediated by β-catenin in the presence of mutp53 (Cagatay and Ozturk, 2000). Furthermore, c-Myc and Oct4 are upregulated in cells that express mutp53, which could be explained by upregulation of β-catenin activity, but additional experimental data are required to demonstrate this signaling axis (Hosain et al., 2016).

Some *in vivo* models have been employed that overactivate Wnt signaling to generate tumors in mice. Wnt-1 transgenic mice develop mammary cancer, but when the mice are additionally modified with a mutant version of p53 (R175H), a higher number of tumors in many mammary glands are observed. Additionally, there is an increase in the pool of mammary epithelial stem cells, which is related to tumorigenic potential (Lu et al., 2013).

Similarly, synergistic oncogenic properties of mutp53 have been shown in two additional mouse models of Wnt-driven gastrointestinal cancer. In these studies, there are exogenous conditions related to microenvironment, such as gut microbiome, that determine the functions of mutp53 in two different anatomical sites, intestine and colon. The microorganisms of the intestinal tract and colon are interacting with the host cells maintaining homeostasis. Recently, it was evidenced that a microbiome imbalance can promote tumorigenesis, and its anatomical localization has proven to be a key factor to promote the oncogenic effect of mutp53. In this study, mutp53 GOF was dependent on gallic acid, a metabolite that simulates the effects of the microbiome in the gut. This became evident because mutp53 strengthened tumor occurrence in distal sites, like colon, characterized by gallic acid enrichment, while mutp53 diminished tumor progression into proximal sites, where gallic acid is scarce. In the proximal site, the tumor-suppressive activity of mutp53 was independent of canonical p53 transcription and more closely associated with suppression of the Wnt pathway. TCF4, the transcriptional factor mediating β-catenin activity, was shown to be decoupled from chromatin and the H3K4me3 active transcription epigenetic marker was dropped from Wnt response targets. Noticeably, this mechanism of Wnt signaling inhibition was not observed in crypts, in the presence of gallic acid, diminishing the protective task of mutp53 against tumorigenesis and conversely, promoting tumor development, as reflected by canonical Wnt targets, such as CD44, c-myc, or Axin2. The overall data postulate a dual role for mutp53, in which the exogenous components and the effects of the microbiome, though gallic acid, might decide the transition from tumor-suppressive to oncogenic function, or vice versa (Kadosh et al., 2020).

The YAP/TAZ complex (Yes-Associated Protein/Transcriptional Co-Activator with PDZ-binding motif), is a key component of mechanical stress. YAP/TAZ complex is important for self-renewal and tumorigenic capacity (Cordenonsi et al., 2011). Escoll et al. (2017) described that mutp53 stimulates YAP/TAZ stability through phosphorylation of WIP (WASP-Interacting Protein) by AKT2, in glial and breast cancer cells. In this context, mutp53 upregulated CSC

marker (CD133 and CD44) expression, sphere formation and tumor capability. Interestingly, in this study mutp53 requires membrane-associated components to transmit the signal to WIP, similar to oncogenic pathway related GOF. This evidence indicates a mechanism by which mutp53 can also regulate CSC self-renewal.

The loss of p53, as well as GOF attributed to mutp53, seems to be necessary for the presence of tumor-initiating properties, as recently described in intestinal cancer. The loss of p53 function in addition to mutp53, promotes a greater ability to form organoids and increases tumorigenic capabilities, since the presence of one functional allele of *TP53* with mutp53 does not increase these characteristics. Particularly, this genotype shows elements related to stemness and inflammatory pathways. This evidence is not trivial, since clonal expansion, survival and metastatic effects are attributed to mutp53 in both alleles (Nakayama et al., 2020).

GAIN-OF-FUNCTION AND CHEMORESISTANCE

Mechanisms of Chemoresistance

Resistance to chemotherapy is a major cause of cancer-associated death that can occur due to several factors, including enhanced drug efflux and metabolic reprograming (Holohan et al., 2013). Evidence indicates that GOF activities promote tumor progression and can drive resistance to a variety of anticancer drugs (**Figure 7**). In fact, many studies show that mutp53 is associated with increased expression of MDR1 (multidrug resistance gene 1), an important drug efflux pump (Sampath et al., 2001). It was discovered that mutp53 knockdown reduces cell proliferation and resistance to cisplatin, adriamycin and etoposide in several cancer cells lines (Bossi et al., 2006). In humans, colorectal carcinomas harbor frequent mutation of *TP53* that are associated with resistance to tyrosine kinase inhibitors (Wang B. et al., 2013).

In a prospective study including patients with locally advanced breast cancer, it was observed that those with mutp53 status treated with doxorubicin and a combination of 5-fluorouracil and mitomycin, showed a reduced recurrence-free and overall survival in comparison to patients with wtp53 tumors (Eikesdal et al., 2014). Moreover, it has been reported that the K351N mutp53 may be associated with induction of platinum resistance in patients with advanced epithelial ovarian cancer (Zhang et al., 2014).

Although impact of mutp53 on drug resistance has been widely reported, it is important to understand the mechanisms through which such resistance is achieved. Some p53 mutants provide enhanced resistance to apoptosis induced by a variety of chemotherapeutic drugs (Wang Q. et al., 2013). Interestingly, it was shown in a squamous cell carcinoma model, that overexpression of the R273H mutant is associated with doxorubicin and methotrexate resistance through the inhibition of apoptosis by procaspase-3 downregulation (Wong et al., 2007). In accordance with this, Donzelli and coworkers (2012) showed that mutp53 also confers chemoresistance to doxorubicin, cisplatin and 5-fluorouracil by procaspase-3 downregulation. Specifically, they demonstrated that the R175H mutp53 is able to induce miR-128-2 expression, which in turn upregulates p21, promoting its accumulation in the cytoplasm favoring cellular survival (Donzelli et al., 2012).

Since DNA damage is one of the central mechanisms of current chemotherapeutic drugs, up-regulation of DNA repair function promotes resistance to these agents. Interestingly, a recent study showed that the mechanism of resistance to adriamycin in breast cancer with mutp53 was through downregulation of miR-30c and translesion synthesis DNA polymerase, REV1, indicating that mutp53 favors the DNA damage repair pathway (Lin et al., 2019). Moreover, Yan et al. (2018) demonstrated that inhibition of UDG (Uracil DNA Glycosylase) selectively sensitized mutp53 cancer cells to 5-FdU (Floxuridine), but did not alter the response in wtp53 cancer cells. Since UDGs are DNA repair enzymes, that also recognize 5-FdU to initiate the base excision repair pathway, these enzymes are important for the effect mediated by 5-FdU. Thus, UDG depletion restores sensitivity and has chemotherapeutic potential in the context of cancer with mutp53. Previous studies have indicated that mutp53 decreases chemosensitivity of glioblastoma to temozolomide by increasing the expression of MGMT (O6-methylguanine DNA-methyltransferase), this enzyme is involved in repair of DNA damage caused by temozolomide, thereby contributing to drug resistance (Wang et al., 2014).

In other studies, it has been shown that several p53 mutants, such as R273H, C176S, and R248W, promote gemcitabine resistance in pancreatic adenocarcinoma cells lines. Specifically, gemcitabine stabilizes mutp53 and promotes its phosphorylation at Ser15, mutp53 then induces CDK1 (Cyclin Dependent Kinase 1) and CCNB1 (Cyclin B1) gene expression promoting a hyperproliferation effect and chemoresistance (Fiorini et al., 2015).

Recently, an effect of mutp53 over EFNB2 (Ephrin-B2), which is a receptor tyrosine kinase that regulates invasion, migration, angiogenesis and tumor resistance has been reported (Zhu et al., 2020). Moreover, it was reported that mutp53 increases EFNB2 expression in colorectal carcinoma cell lines, when the cells were treated with 5-FU (Alam et al., 2016). EFNB2 induces 5-FU resistance through the upregulation of the ABCG2 (ATP-binding Cassette Sub-family G-2) multi-drug resistance efflux transporter, mediated by the activation of the c-Jun/JNK signaling pathway (Alam et al., 2016). Recently, it was observed that mutp53 upregulates the receptor tyrosine kinase AXL in colon and breast cancer models, and also contributes to the EMT process, impairing the response to therapy. Interestingly, expression of AXL confers an invasive potential to mutp53 cells after exposure to chemotherapy, compared to cells where AXL was silenced. In support of this, mutp53 increases AXL expression at mRNA and protein levels (Zhu et al., 2019).

The survival of cancer cells can be modulated by Ca^{2+} dynamism, which represents an important influence on chemoresistance. The excessive transfer of Ca^{2+} to the mitochondria drives pro-apoptotic responses. Interfering with this process improves mechanisms related with cellular survival, including autophagy. The role of Ca^{2+} depends

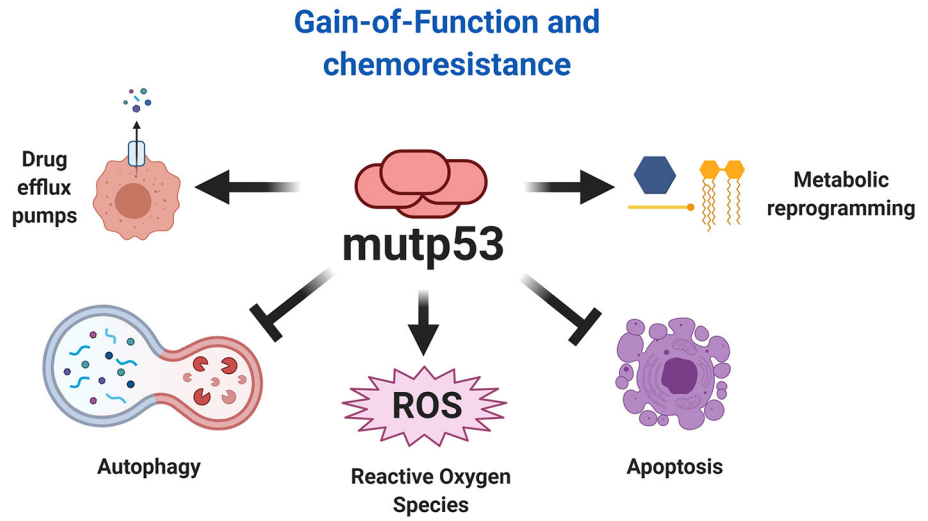

FIGURE 7 | Chemoresistance explained by mutp53. The gain-of-function of mutp53 has been broadly implicated in various mechanism of chemoresistance, including resistance to apoptosis, autophagy inhibition, metabolic reprogramming, and increased expression of drug efflux pumps. Additionally, the effect of mutp53 and the downstream effects of gain-of-function favors autophagy inhibition and ROS production, being an important mechanism that explains the overactivation of oncogenic pathways.

on p53 functionality. Wtp53 increases Ca^{2+} transfer from endoplasmic reticulum to mitochondria, promoting apoptosis under stress conditions (Bittremieux and Bultynck, 2015). In the absence of p53 and the presence of mutp53 (R175H, R273H), cancer cells fail to transfer Ca^{2+} to the mitochondria, favoring chemoresistance to stressor treatments (Giorgi et al., 2015). This mechanism can be useful in therapy to control the effect of pharmacological treatments.

It was reported that p53 mutants establish amyloid-like aggregates that contribute to cancer progression and tumor resistance (Levy et al., 2011; Yang-Hartwich et al., 2015). These aggregates sequester native p53 protein into an inactive conformation lacking pro-apoptotic function, leading to platinum resistance in high-grade serous ovarian carcinoma cells (Yang-Hartwich et al., 2015). More importantly, mutations in p53 may shift the conformation distribution favoring the generation of aggregates. For instance, a recent study found the presence of amyloid-like mutant p53 in brain tumor cells showing chemoresistance to temozolomide (Pedrote et al., 2020). Interestingly, Zhang et al. (2017) identified ERP29 (endoplasmic reticulum protein 29) as a key mediator of chemoresistance by the aggregation prone R282W mutp53 and suggest that targeting ERP29 may sensitize cancer cells to cisplatin treatment. They show that R282W mutp53 upregulates ERP29 at the mRNA and protein expression levels. Conversely, using ReACp53, a peptide inhibitor for p53 aggregation, they observed a decrease in ERP29 and ultimately the reversion of chemoresistance.

Recently, it was shown that the R273H mutant induces 5-FU resistance in colorectal cancer through the downregulation of the proapoptotic protein PUMA. Employing ChIP assays, they show that mutp53 could not bind to the PUMA promoter, which impairs its transcription. PUMA is a critical upstream activator of the proapoptotic protein BAX, thus mutp53 decreases BAX activity and hence the apoptotic process (Huang et al., 2019). The fact is that several reports show that PUMA induction by chemotherapeutic agents and adenoviral delivery assays, suppresses tumor growth and sensitizes to chemotherapy through induction of apoptosis in head and neck cancer (Sun et al., 2007).

Nuclear effects of mutp53 can increase chemoresistance, since mutp53 can act with the YAP/β-arr1 complex to improve its transcriptional program over the TEAD transcription factor. This effect seems to be explained by the ET-1R (Endothelin-1 Receptor) signaling pathway, a mechanism related to the increase in YAP activity. This process promotes resistance to cisplatin, as was recently described in ovarian cancer. The use of ET-1R antagonists, such as macitentan, affects the nuclear YAP/mutp53/β-arr1 complex, making cancer cells more sensitive to cisplatin and representing an important axis that can be disrupted in patients with this type of cancer. This is not just another mechanism, since it offers an explanation of where mutp53 pathways interconnect, for improve chemoresistance (Tocci et al., 2019).

Autophagy Inhibition by Mutant p53

The role of autophagy in cancer depends of the types of oncogenes observed, which offers interesting views, from a metabolic perspective, related to chemoresistance (White, 2016). The GOF of mutp53 promotes autophagosome inhibition. However this process is much more complex when we try to understand the metabolic consequences related to autophagy inhibition, since in different types of cancer "autophagy addiction" is necessary for tumor cell survival (Santana-Codina et al., 2017).

Autophagy is a catabolic process where macromolecules and organelles are degraded into lysosomes, providing energetic fuels under stress conditions (Santana-Codina et al., 2017). Autophagy is determinant for the progression of some types of cancer. For example, continuous proliferation is a characteristic of Ras-driven cancer types, in which intracellular autophagy markers are increased, providing biosynthetic precursors, intermediaries of TCA (tricarboxylic acid) cycle, and nucleotides critical for growth and survival during stress conditions, such as starvation (Guo et al., 2011, 2016; Yang et al., 2011). In this sense, pancreatic ductal adenocarcinoma tissues and cell lines reveal increased autophagosomal markers, which is determinant for malignant progression, since autophagy inhibition compromises growth and tumorigenicity (Yang et al., 2011).

There is a relationship between autophagy and p53 functions, since wtp53 induces autophagy and conversely, autophagy inhibits wtp53 activity. Moreover, wtp53 functions favor autophagy through transcriptional response, this process ensures cellular homeostasis under stress damage. In the case of cancer, wtp53 can limit oncogenic transformation and autophagy driven by K-Ras (White, 2016). Thus, oncogenes and tumors suppressor genes can influence the role of autophagy in cancer.

The increases in autophagy flux represents an important element in the development and progression of Ras-driven cancer types. However, this is not the case when there is a loss of p53 function. Interestingly, in cell line and mice models that harbor mutated K-Ras, it has been reported that autophagy is not active under conditions where p53 is not functional, resulting in an important decrease of autophagy protein markers as well as autophagosomes. Interestingly, Rosenfeldt et al. (2013) observed that autophagy inhibition is crucial for pre-cancerous pancreatic intraepithelial neoplasia and favors pancreatic adenocarcinoma, potentiating tumor cell growth, even more than the autophagy induced by K-Ras. This evidence allows us to establish that pharmacological inhibition of autophagy in cancer may be counterproductive. Therefore, anabolic requirements generated by the absence of wtp53 must be considered. In the case of Ras-driven cancer, the requirement for oxidative metabolism through mitochondrial function, as determined by oxygen consumption, along with the ATP and macromolecule biosynthesis requirements, explain the "autophagy addiction," so these types of cancers are more sensitive to autophagy inhibitors (Guo et al., 2011, 2016; Yang et al., 2011).

In tumor cells with non-functional p53, metabolic requirements seem to be a consequence of the Warburg effect, since the dual effect of a K-Ras mutation and absence of wtp53 increases glucose uptake, releasing lactate as a metabolic precursor to sustain anabolic pathways, while oxygen consumption or synthesis of TCA cycle intermediaries are not affected. Autophagy inhibition combined with the absence of p53 increases the tumorigenic capabilities of K-Ras driven cells, as determined by lower overall survival of mice with pancreatic adenocarcinoma (Rosenfeldt et al., 2013; White, 2016).

The status of p53 is determinant for the correct use of autophagy flux inhibitors as a tool that could compromise the proliferative capacities of cancer cells, since autophagy inhibition can increase premalignant lesions in pancreas due to Ras oncogene and loss of p53, but the capacity to acquire an invasive phenotype is impaired. Yang et al. (2014) argue that autophagy inhibition can be employed independently of TP53 status, reducing the oxygen consumption rate and clonogenicity (Yang et al., 2011, 2014; Rosenfeldt et al., 2013). Similarly, it has been suggested that the reestablishment of canonical functions of wtp53 on tumor cell lines with different TP53 mutants, using reactivator molecules, could sensitize cancer cells to apoptosis. It has been observed that dual administration of these treatments with autophagy flux inhibitors have important effects for reducing the proliferative capability. However, this effect is not observed with the individual use of wtp53 reactivator molecules. This leads to positioning autophagy as a survival process, so that unique treatments based on reestablishing wtp53 seem to be therapeutically unviable (Fiorini et al., 2013).

Determination of mutp53 is critical for elucidating the differential effects observed over autophagy. While mutp53 proteins that localize to the nucleus enhance autophagy, those that localize to the cytoplasm decrease it (Morselli et al., 2008). One of the proposed mechanisms explaining the downregulation of autophagy related to GOF is through autophagosome protein markers, like Atg12 and Beclin1, among others. The expression of autophagosomal markers is NF-κB dependent, however, mutp53 binds to p50 (NF-κB subunit), promoting repression of Atg12 expression. In agreement with other reports, mutp53 downregulates AMPK activation, which is reflected by an increase in mTORC1 activity, a suppressor of the autophagy process. Taken together, these reports sustain a molecular basis by which autophagy is reduced in the presence of mutp53. Interestingly, different cancer cell lines with mutp53 are more sensitive to inhibition of mTORC1 (Zhou et al., 2014; Cordani et al., 2016).

The inhibition of mTORC1 could generate an increase in autophagic flux and consequently contribute to pharmacological resistance. For example, it was recently shown that overexpression of miR-338-3p confers 5-FU (5-fluorouracil) resistance in colorectal cancer cells with p53 mutant status by targeting the mTORC1 pathway (Han et al., 2017). Consequently, mTOR impairment resulted in an increase in autophagy. They showed that the mechanism influencing 5-FU sensitivity was due to competition between autophagy and apoptosis. In this case, autophagy could be playing a key role in protecting cancer cells from stress-induced damage caused by 5-FU (Han et al., 2017).

THERAPEUTIC PERSPECTIVES

Mutp53 GOF is observed in most human cancers and generates a dependence for tumor maintenance by several mechanisms shown in **Table 1**. The inhibition of mutp53 represents an effective strategy for therapy. Currently, there are different therapeutic strategies focused on targeting mutp53. We have decided to classify them into three categories: restoring wtp53 functions, disrupting REDOX balance, and targeting mutp53 for degradation (Mantovani et al., 2019; Zhang C. et al.,

TABLE 1 | Mutant p53 gain-of-function.

Gain-of-function	Molecular mechanism	Mutant version	Type of cancer	References
Proliferation	Increasing receptors translocation through the RCP complex	R175H R273H	Breast cancer	Muller et al., 2009
	Increasing the PI3K/AKT axis through inhibition of DAB2IP	R280K R175H	Breast and prostate cancer	Valentino et al., 2017
	Increasing the active state of K-Ras	R175H	Pancreatic cancer	Escobar-Hoyos et al., 2020
Migration and metastasis	Interacting with p63 and downregulating its anti-metastatic activities	R175H	Breast cancer	Gaiddon et al., 2001; Adorno et al., 2009
	Increasing Rac1 activity	R175H R248W R273H	Colorectal cancer	Yue et al., 2017
	Interacting with HIF1α and favoring secretome activity and metastatic capabilities	R175H R273H R280K	Breast cancer	Capaci et al., 2020
Metabolic reprogramming	Increasing activity of RhoA/ROCK axis and translocation of glucose transporters to membrane	R175H R248Q R273H	Lung and breast cancer cells	Zhang et al., 2013
	Interacting with SREBP and increasing the MVA pathway	R273H	Breast cancer	Freed-Pastor et al., 2012
	Inhibiting AMPK activity and increasing anabolic pathways	R175H G245C R282W	Head and neck squamous cell carcinoma	Zhou et al., 2014
Immune evasion	Increasing inflammation and favoring NF-kB activity	R273H	Colorectal cancer.	Cooks et al., 2013
	Augmenting pro-inflammatory activity in the tumor microenvironment by interaction with MAFF	R273H	Colon and breast cancer cells	Ubertini et al., 2015
	Reprogramming macrophages from M1 to M2	R245 R248 R175 R273 R282	Colorectal cancer	Cooks et al., 2018
Stemness	Increasing CSC surface markers and ALDH enzymatic activity	R175H R273H	Colorectal cancer	Solomon et al., 2018
	Increasing activity of YAP/TAZ pathways and promoting self-renewal of CSC	R175H R273H	Glioblastoma and breast cancer cells	Escoll et al., 2017
	Inducing a repressive state of chromatin through PRC2 activity	R175H R248W R273H	Hematopoietic stem cells	Chen et al., 2019
Chemoresistance	Favoring changes in transcriptional regulation by mutp53/YAP/β-arr1 and promoting cisplatin resistance	R273H	Ovarian cancer	Tocci et al., 2019
	Up-regulating DNA repair pathways	R280K	Breast cancer	Lin et al., 2019
	Downregulating procaspase-3 by increasing miRNA-128-2	R175H	Non-small-cell lung cancer	Donzelli et al., 2012

2020). Based on this, there are several clinical trials registered (ClinicalTrials.gov), and some of them use these strategies in combination with common chemotherapeutic treatments to prevent resistance to the current therapy.

To Restore or Not to Restore *Wild Type* p53 as Therapy

Therapeutic strategies for treatment of tumors with mutp53, include reestablishing normal p53 functions. Presence of missense mutations in p53 destabilizes the zinc interaction, resulting in misfolding and loss of a *wild type* tridimensional structure (Joerger and Fersht, 2008). Interestingly, supplemental zinc in culture media has been shown to restore *wild type* structure in some mutp53 cells, as well as its corresponding transcriptional activity (Margalit et al., 2012). Innovatively, it was found that treatments such as Zn-cur (Zinc-curcumin complex) induce a structural change from mutp53 to wtp53, restoring its canonical functions. This type of treatment can even cross the blood-brain barrier, and in the case of the glioblastoma model could be an important treatment strategy (Garufi et al., 2013).

Similarly, another metal ion chelator is COTI-2, which can bind to misfolded mutp53 forms and restore p53 activity as a transcription factor, leading to reactivation of wtp53 target

genes such as p21, PUMA, and NOXA. COTI-2, has been tested in preclinical phase studies on different types of cancer (Lindemann et al., 2019). For instance, one clinical trial has used it as monotherapy or combined with cisplatin in lung and colon cancer patients with recurrent malignancies (NCT02433626).

Another proposal is the use of PRIMA-1 and its analog ARP-246, whose mechanisms of action is through the MQ (methylene quinuclidinone) metabolite. Mutp53 can recover its transcriptional activity through the generation of adducts, which increase DNA-binding, through an alkylation process that favors its correct folding. Once inside the cell, PRIMA-1 is degraded to MQ, and interacts with the thiol chemical groups of mutp53, thus promoting recovery of its transcriptional activity (Lambert et al., 2009).

APR-246 is considered to be a promising first-in-class mutp53 targeting drug, since it is more potent and less toxic than PRIMA-1 (Lambert et al., 2009). Currently, there are ten clinical trials in phase I and II for different types of cancer registered at ClinicalTrials.gov (NCT03268382, NCT02098343, NCT00900614, NCT04214860, NCT04383938, NCT03072043, NCT03588078, NCT03745716, NCT03391050, NCT04419389). The combined use of APR-246 and Pegylated Liposomal Doxorubicin Hydrochloride (PLD) has been used on ovarian cancer patients with mutp53 (NCT03268382 and NCT02098343), however the effectiveness is not yet well defined.

Other report supports that acetylation of mutp53 (R158G) through pharmacological agents alters its ability to bind DNA, decreases the oncogenic effect of NF-kB activity, and finally, favors apoptosis, making cancer cells sensitive to DNA-damaging agents. The particular effects of mutant p53 versions can be employed for the use of specific treatments focused on their GOF (Kong et al., 2020).

Reports that focus on reestablishing wtp53 functions have determined that PEITC (Phenethyl isothiocyanate) can favor canonical p53 activity in cell line models expressing endogenous mutp53, by promoting a wtp53-like conformational state. This effect recapitulates the ability to be regulated by ubiquitination and even to reduce the tumorigenic ability observed in xenograft models (Aggarwal et al., 2016, 2019).

Recently, it has been shown that wtp53 improves metabolic adaptation and favors tumor progression by PUMA regulation, a proapoptotic protein overexpressed in hepatocellular carcinoma, and by increasing mitochondrial activity through oxidative phosphorylation (Bensaad et al., 2006; Puzio-Kuter, 2011). However, overexpression of PUMA and wtp53 phosphorylation by IKKβ promotes its binding to MPC (Mitochondrial Pyruvate Carrier), which impairs the transport of pyruvate to the mitochondria. The functional relevance of this mechanism is to drive a preponderantly glycolytic pathway, avoiding oxidative phosphorylation as a major fuel for ATP. This supports the survival process related with wtp53 in this type of model. The effect of wtp53 over PUMA can act as an oncogenic axis, since wtp53 or PUMA knockdowns can preclude, not only this metabolic switch, but also tumorigenic capability, making it challenging to restore wtp53 functions with the purpose of therapy (Kim J. et al., 2019).

New reports reveal that in cancers expressing wtp53, such as melanoma, its overactivation favors a slow-cycling phenotype, a population characterized by low proliferation but higher invasive capabilities that result in pharmacological resistance. Increasing wtp53 induces cellular arrest and senescence, but not a proapoptotic effect, suggesting a dynamism favoring survival. In this case, impairing wtp53 together with the use of MAPK inhibitors could be important to reduce the metastatic capabilities of melanoma cells. This evidence suggests an alternative role for wtp53 that drives chemoresistance (Webster et al., 2020).

Another report sustains that under stress conditions caused by low availability of glutamine, the transcriptional activity of wtp53 and mutp53 can be restored through phosphorylation by IKKβ, regulating genes related with survival of cancer cells, while proapoptotic genes are not expressed (Ishak Gabra et al., 2018). This evidence allows us to establish a solid molecular base by which the reestablishment of wtp53 canonical functions could have counterintuitive effects on metabolic adaptation. This suggests that the use of treatments based on improving wtp53 function is not the only answer, and it is necessary to define the p53 functional status in cancer in order to understand the possible mechanisms that could result in pharmacological resistance.

Disrupting the Redox Balance

As a consequence of the presence of mutp53, ROS accumulates due to both, loss of wtp53 antioxidant capacities and mutp53 GOF. This includes antioxidant enzyme imbalance, inhibition of autophagy, and the overactivation of oncogenic pathways (Cordani et al., 2020).

It is important to highlight that the hyperproliferation process related to mutp53 involves an increase in ROS. Simultaneously, the presence of mutp53 suppresses antioxidant responses through the repression of Nrf2 activity, as well as glutathione synthesis, both implied in the improvement of antioxidant mechanisms (Liu D. S. et al., 2017). One of the goals of preventive treatment is the upregulation of canonical p53 functions, with the purpose of favoring antioxidant capabilities as a protective tool for responses such as DNA damage. In this manner, in the face of oncogenic damage it would be generating apoptosis or cell cycle arrest (Budanov, 2014). For example, employing the natural phenolic compound curcumin, an antioxidant agent with anti-inflammatory properties, there was an increase in p53 half-life, given by interaction with the NQO1 protein. This mechanism is an important element to improve the decrease in cell viability observed in cell lines with wtp53 (Patiño-Morales et al., 2020).

However, continuous treatment to reestablish the p53 functions could result in pharmacological resistance, since the increase in antioxidant activity in cancer cells would prevent cell death caused by exogenous stress generated by radiotherapy or the oxidant stress of a chemotherapeutical treatment (Conklin, 2004; Trachootham et al., 2009). Moreover, increasing antioxidant ability protects against ROS through

wtp53, but may lead to apoptosis resistance. Additionally, p21, a transcriptional target of wtp53 can promote mechanisms related to survival, such as senescence. Therefore, one must be careful when using pharmacological treatments for different types of cancer where the functional state of p53 is not well known. The importance of REDOX balance could be employed to compromise mutp53 function (Vousden and Prives, 2009).

For instance, the hypoxia process favors cell cycle arrest and pharmacological resistance, since it impairs the proliferation process as well as apoptosis induced by generation and accumulation of ROS. The accumulation of oxidative stress with APR-246, a treatment able to reactivate wtp53 functions, makes mutp53 cells more sensitive to apoptosis cell death. Antagonizing ROS accumulation with N-acetyl cysteine avoids cell death under these conditions (Deben et al., 2018). This evidence highlights the importance of REDOX balance, since cancer cells, upon losing their antioxidant capacity, sensitize cells to death by ROS accumulation, which can be employed to compromise mutp53 cancer cells leading to apoptosis.

Cordani et al. (2018) observed that ROS accumulation in cells with mutp53 improves mitochondrial membrane potential without affecting mitochondrial DNA. However, the addition of an oxidant stressor such as H_2O_2, makes cancer cell lines with different p53 mutants more sensitive to apoptosis. These authors propose that the Achilles heel that compromises mutp53 is based on REDOX balance (Cordani et al., 2020). This process could be employed for the implementation of therapies that allow a decrease in mutp53 GOF through ROS generation.

Additionally, these authors reveal that AMPK inactivation driven by "hotspot" mutp53 can be through different mechanisms, including sestrin inhibition. Sestrins are enzymes that bind directly to AMPK and promote its activation (Cordani et al., 2016). Importantly, AMPK inactivation reduces the activity of PGC-1α and UCP2, both proteins implicated in ROS-scavenging. The pro-oxidant response caused by mutp53 can be used to compromise the functions of mutp53. Therefore, intracellular ROS accumulation can represent an important feature to explain the genomic instability generated by mutp53 (Cordani et al., 2018). However, cancer cells with mutp53 restrict the levels of ROS to avoid the cytotoxic effects caused by an oxidant state, as was recently described. Mutp53 (R273H) increases the expression and activity of antioxidant protein MnSOD (Manganese Superoxide Dismutase) and SIRT3, preventing the harm exacerbated by ROS (Torrens-Mas et al., 2020). This evidence reveals a novel mechanism that confers cancer cells with mutp53 protection to avoid ROS damage. Thus, the cytotoxicity threshold dictated by ROS, which distinguishes between evading or promoting apoptosis, can be useful for therapy, as shown in **Figure 8**.

Moreover, it has been proposed that the inhibition of antioxidant capacity, along with prooxidant drugs (like doxorubicin or cisplatin), could sensitize cancer cells to apoptosis. However, the therapeutic feasibility of prooxidant drugs in cancer with mutp53 status is not well defined (Kim S. J. et al., 2019).

Targeting mutp53 Stability to Decrease GOF

An attractive alternative for therapy involves the targeted degradation of mutp53 protein to reduce its half-life (Freed-Pastor et al., 2012). As mentioned earlier, some p53 mutant proteins, in addition to not being functional, generate aggregates.

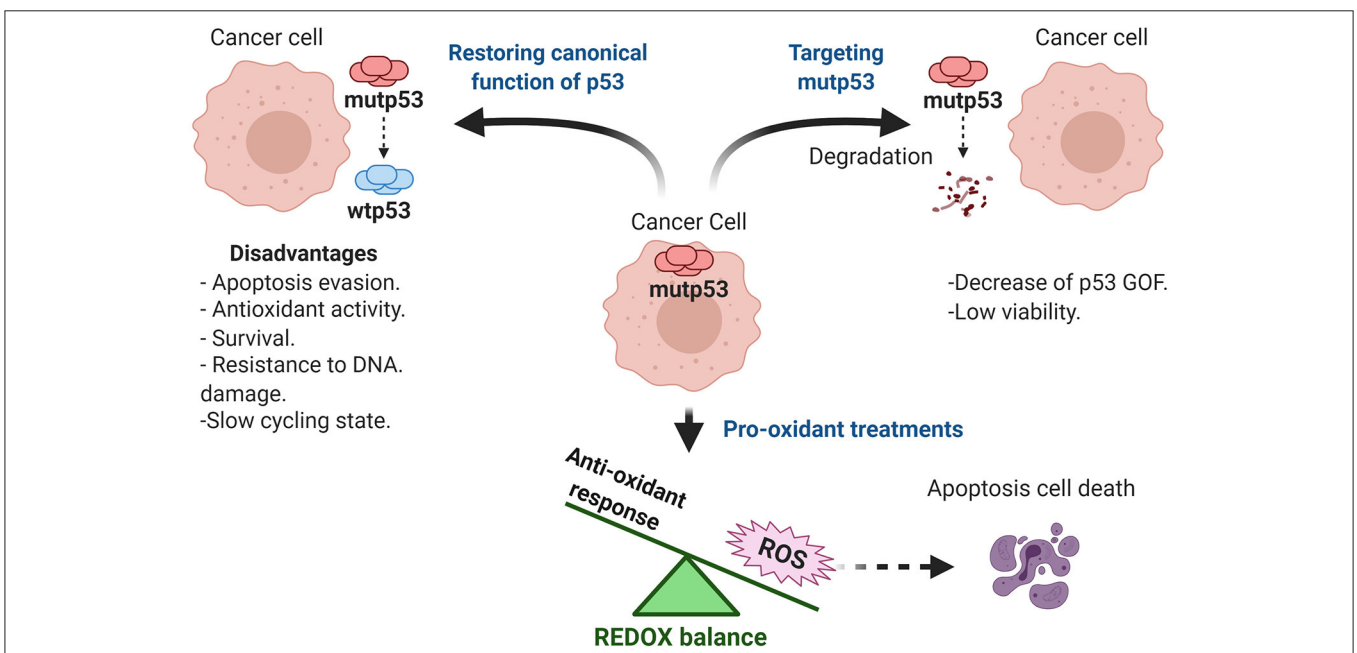

FIGURE 8 | Therapeutic approaches targeting mutp53. Therapeutic strategies for treatment of tumors with mutp53 include reestablishing canonical functions of wild type p53, blocking mutp53 GOF, as well as pro-oxidant treatments.

Under hypoxic conditions, autophagy has been shown to promote degradation of such aggregates through the CHIP protein (C terminus of Hsc70-interacting protein), suggesting it can be a key element to selectively reduce mutp53 GOF under hypoxic conditions (Maan and Pati, 2018).

Accumulation of mutp53 proteins can be regulated through inhibition of the MVA pathway. The accumulation of mutp53 is given by their interaction with several HSP (heat shock) proteins. Their inhibition would not only impact signaling pathways that are dependent on lipogenic routes, but also by the stability of mutp53. Using chemical libraries it has been observed that statins decrease viability of cell lines with mutp53, and that this effect is due to the ubiquitination and degradation of mutp53 through the proteasomal pathway (Parrales et al., 2016; Ingallina et al., 2018). The effects of statins on viability of null or *wild type* p53 cell lines were minimal, supporting their potential role as a viable pharmacological strategy for different types of cancer with mutp53 (Chou et al., 2019). The use of statins has been repositioned for use in cancer treatment. For instance, atorvastatin is actually in phase I of different clinical trials. The use of atorvastatin as monotherapy or combined is currently being studied in acute myeloid leukemia and breast cancer with p53 mutations (NCT0356088 and NCT03358017).

Another proposed strategy is based on degradation of p53 HDAC (Histone deacetylase) whose functions are not limited to histones, but also regulate activity of transcription factors, including p53 (Yan et al., 2013). Inhibition of HDAC using SAHA (suberoylanilide hydroxamic acid) impairs the interaction of mutp53 with HSPs, favoring the interaction with Mdm2 and CHIP, and thereby increasing its degradation (Meng et al., 2018).

CONCLUSIONS

Considering the old and new findings related to mutp53 GOF, many of the intracellular pathways of cancer cells can be explained by p53 status. However, there are challenges that need to be answered such as the complete functions of wtp53, the new mechanisms related to GOF, the adaptation of mutp53 to stress conditions, as well as the influence of mutp53 over immune system cells. This makes it difficult to provide easy solutions for a disease that is not completely understood. The translational focus of this exciting field of knowledge in different types of cancer and, the presence of mutp53 proteins is certainly an important and sometimes crucial driving force in the human tumors harboring them. It is noteworthy that mutp53 proteins affect central cellular and tissue processes and systems that include stemness, immune evasion, metabolic control, migration, proliferation, explaining the biology of cancer cells. The understanding of the molecular bases for mutp53 GOF will hopefully allow us to establish common mechanisms for different cancers, not limited to those that harbor mutp53. Currently, the knowledge of p53 status (wild type or mutant proteins) constitutes a crucial factor for the correct use of anti-tumor treatments. This knowledge will allow a better understanding of multiple processes involved in the behavior of cancer cells, including chemoresistance, immune evasion, promotion of stemness, apoptosis resistance, metabolic reprogramming and autophagy, which can help improve current cancer treatments.

AUTHOR CONTRIBUTIONS

EA-O and AG-C conceived this review. EA-O and KC-L drafted the initial version with support of JB-R and MS-S who performed the bibliographical search. EO-S reviewed the manuscript and suggested modifications. AG-C supervised, enriched and corrected different versions of the manuscript whose final version was approved by all authors.

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The Gain-of-Function p53 R248W Mutant Promotes Migration by STAT3 Deregulation in Human Pancreatic Cancer Cells

*Luisa Klemke[1], Clara F. Fehlau[1], Nadine Winkler[1], Felicia Toboll[1], Shiv K. Singh[2], Ute M. Moll[3] and Ramona Schulz-Heddergott[1]**

[1] Institute of Molecular Oncology, University Medical Center Göttingen, Göttingen, Germany, [2] Department of Gastroenterology, Gastrointestinal Oncology and Endocrinology, University Medical Center Göttingen, Göttingen, Germany, [3] Department of Pathology, Stony Brook University, Stony Brook, NY, United States

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***Correspondence:**
Ramona Schulz-Heddergott ramona.schulz@zentr.uni-goettingen.de

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Missense p53 mutations (mutp53) occur in approx. 70% of pancreatic ductal adenocarcinomas (PDAC). Typically, mutp53 proteins are aberrantly stabilized by Hsp90/Hsp70/Hsp40 chaperone complexes. Notably, stabilization is a precondition for specific mutp53 alleles to acquire powerful neomorphic oncogenic gain-of-functions (GOFs) that promote tumor progression in solid cancers mainly by increasing invasion and metastasis. In colorectal cancer (CRC), we recently established that the common hotspot mutants mutp53^{R248Q} and mutp53^{R248W} exert GOF activities by constitutively binding to and hyperactivating STAT3. This results in increased proliferation and invasion in an autochthonous CRC mouse model and correlates with poor survival in patients. Comparing a panel of p53 missense mutations in a series of homozygous human PDAC cell lines, we show here that, similar to CRC, the mutp53^{R248W} protein again undergoes a strong Hsp90-mediated stabilization and selectively promotes migration. Highly stabilized mutp53 is degradable by the Hsp90 inhibitors Onalespib and Ganetespib, and correlates with growth suppression, possibly suggesting therapeutic vulnerabilities to target GOF mutp53 proteins in PDAC. In response to mutp53 depletion, only mutp53^{R248W} harboring PDAC cells show STAT3 de-phosphorylation and reduced migration, again suggesting an allele-specific GOF in this cancer entity, similar to CRC. Moreover, mutp53^{R248W} also exhibits the strongest constitutive complex formation with phosphorylated STAT3. The selective mutp53^{R248W} GOF signals through enhancing the STAT3 axis, which was confirmed since targeting STAT3 by knockdown or pharmacological inhibition phenocopied mutp53 depletion and reduced cell viability and migration preferentially in mutp53^{R248W}-containing PDAC cells. Our results confirm that mutp53 GOF activities are allele specific and can span across tumor entities.

Keywords: mutant p53, missense p53 mutant, STAT3, selectivity, specificity, PDAC, GOF, Hsp90

INTRODUCTION

Already in the early 1990s, the tumor suppressor p53 was coined as 'guardian of the genome' (1, 2) and it was known that mutation of the *TP53* gene (tumor protein p53, HGNC:11998) is an essential step in human tumor development (1, 3). Ever since, scientists have tried to understand the influence of the *TP53* status within the mutational landscape in different cancer entities and to investigate the role of different variants in tumorigenic pathways. It became evident that some p53 mutant protein variants do not only abrogate tumor suppressor functions (loss-of-function, LOF) but also gain new tumorigenic functions (gain-of-function, GOF). Given that approx. 70% are missense mutations leading to amino acid substitutions mostly in the DNA binding domain, some alleles are selected and occur at a high frequency, termed hotspots. Most hotspot mutants gain neomorphic tumorigenic functions, particularly in invasion and metastasis of solid tumors (4–9). A key prerequisite for the GOFs of some missense p53 mutants (termed here 'mutp53') is protein stabilization through the Hsp90/Hsp70/Hsp40 (heat shock protein 90/70/40) chaperone machinery, resulting in protection from MDM2 (mouse double minute 2) and other E3 ligases and thus proteasomal degradation (5, 10–15).

Due to the heterogeneity of *TP53* point mutations, whose phenotypes in addition are highly dependent on the cellular context, different missense mutants exert different cellular responses (16–18). Thus, it is important to consider the context- and allele-dependent specificity of different mutp53 proteins (16, 19–21). To investigate the mutp53 specificity, different groups have dissected the impact of various mutp53 GOF alleles on tumorigenesis using autochthonous mouse models (22–26) or clinical correlation studies (26–29). Recent results from our group highlight the GOF hotspot mutp53$^{R248Q/W}$ specificity in mouse and human colorectal cancer (CRC). mutp53$^{R248Q/W}$ binds to and deregulates phosphorylated STAT3 (signal transducer and activator of transcription 3) by protecting it from SHP2 phosphatase (*PTPN11*, protein tyrosine phosphatase non-receptor type 11), its major negative regulator. Thus, depletion of mutp53$^{R248Q/W}$ inhibits STAT3 signaling and causes suppression of tumor invasion and proliferation (26). The p53 R248 hotspot is the single most common variant in all *TP53*-altered tumor types occurring in 9% of cases, which translates to about 66,000 newly diagnosed cancer patients in the US per year harboring R248 variants. Of R248 substitutions, over 90% are either Q or W, with similar frequencies (The Cancer Genome Atlas Program – National Cancer Institute).

Here, we asked whether mutp53^{R248W} also exhibits tumor-promoting functions affecting migration in pancreatic ductal adenocarcinoma (PDAC). Note that the *TP53*^{R248Q} allele is not available in established PDAC lines. PDAC is currently the fourth leading cause of cancer death worldwide with a rapidly ascending trajectory, and the incidence is predicted to increase even further in the future (30, 31). PDAC, which constitutes around 90% of all pancreatic malignancies, is highly aggressive and chemoresistant and still has a dismal 5-year survival rate of only approx. 9% (30, 32–34).

In approx. 70% of PDAC patients, *TP53* undergoes mainly missense mutations (www.cbioportal.org) as a late genetic event at the transition from high grade PanIN dysplasia to invasiveness during pancreatic cancer progression (35, 36). Here, we show in a panel of common human PDAC cell lines harboring different homozygous missense p53 mutants that mutp53 variants differ in their protein stability, with mutp53^{R248W} again accumulating the highest protein levels also in the pancreatic cell context. Importantly, comparing all PDAC lines, only mutp53^{R248W} depletion strongly reduced migration capacity. In support, mutp53^{R248W} specifically showed the strongest binding to phosphorylated STAT3 under baseline and cytokine-stimulated conditions, forming a constitutive mutp53^{R248W}-pSTAT3 complex. Only mutp53^{R248W} depletion was able to reduce pSTAT3 levels. Consequently, targeting the tumor-promoting mutp53^{R248W}-pSTAT3 complex by pSTAT3 depletion or pharmacological inhibition diminished cell viability and migration in mutp53^{R248W} expressing, but not in mutp53^{R273H} or mutp53^{R282W} expressing PDAC cells. Our results support a GOF function of mutp53^{R248W} in pancreatic cancer cell lines, justifying future investigations in this tumor entity *in vivo*.

MATERIAL AND METHODS

All materials used and corresponding information are provided as **Supplementary Table 1**.

Cell Culture

Homozygous mutant human pancreatic cancer cell lines MIA-PACA-2 (mutp53$^{R248W)}$ (DZMS, RRID : CVCL_0428), PANC-1 (mutp53^{R273H}) (ATCC, RRID : CVCL_0480), BXPC-3 (mutp53^{Y220C}) (ATCC, RRID : CVCL_0186), and PA-TU-8902 (mutp53^{C176S}) (DSMZ, RRID : CVCL_1845) were grown in DMEM (Gibco) with 10% FBS (Merck). PA-TU-8988T (mutp53^{R282W}) (DSMZ, RRID : CVCL_1847) were grown in DMEM medium with 5% FBS. CAPAN-1 (mutp53^{A159V}) (ATCC, RRID : CVCL_0237) were grown in RPMI 1640 (Gibco) with 20% FBS, and L3.6pl cells (truncating frameshift p53 mutation) (37, 38) were grown in RPMI 1640 with 10% FBS. All media were supplemented with Penicillin-Streptomycin (10,000 U/mL, Gibco) and L-Glutamine (Gibco). All cell lines were grown at 37°C at 5% CO_2 in a humidified atmosphere and tested for mycoplasma contamination on a regular basis (Mycoplasma Detection Kit, Lonza). Cell line authentication certificates are provided as **Supplemental Material**.

Transfection With siRNA

Depletion of human *TP53* or *STAT3* mRNAs was achieved by siRNA transfection using Lipofectamine™ 3000 (Invitrogen) or Lipofectamine™ 2000 (Invitrogen) transfection reagents. siRNA sequences are listed in supplemental **Supplementary Table 1**. Cells were reverse transfected in 6-well plates (Sarstedt) according to manufacturer guidelines. After 24 h, supernatant was collected and replaced by fresh culture medium. Seventy-two-hour post-transfection cells were harvested for analyses.

Immunoblot Analysis

Cell lysates were prepared with RIPA buffer containing 20 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% sodium deoxycholate, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, phosphatase inhibitor consisting of 2 mM imidazol, 1 mM sodium orthovanadate and 1 mM sodium fluoride, and cOmplete™ mini protease inhibitor cocktail (Roche). Samples were lysed in RIPA buffer with sonication. Protein concentrations were determined by BCA protein assay (Pierce). Equal amounts of lysates were loaded (15–30 µg) and separated by SDS-polyacrylamide gel electrophoresis followed by transfer onto nitrocellulose membranes (Amersham). After blocking with 5% milk (Roth), membranes were incubated with the following antibodies: HSC70 [B-6] (Santa Cruz), beta-Actin (Abcam), total-AKT [D9E] (Cell Signaling), p53 [DO-1] or HRP-conjugated p53 [DO-1] (Santa Cruz), phospho-Y705 STAT3 [EP2147Y] (Abcam), total STAT3 (Santa Cruz) or total STAT3 [79D7] (Cell Signaling), MDM2 [IF-2] (Calbiochem®/Millipore), p21 Waf1/Cip1 [12D1] (Cell Signaling). Primary antibodies were detected with HRP-conjugated secondary antibodies. Signal was developed using Clarity Max™ Western ECL Substrate (BioRad), SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific), or Immobilion Western chemiluminescent HRP substrate (Millipore/Merck). For antibody details, see **Supplementary Table 1**.

Co-Immunoprecipitation

For coIP, cells were lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet™ P40, 10 µM MG-132, phosphatase inhibitor consisting of 2 mM Imidazol, 1 mM sodium orthovanadate, and 1 mM Sodium Fluoride, and cOmplete™ mini protease inhibitor cocktail (Roche), followed by sonication. After centrifugation, samples were precleared with protein G Sepharose (GE Healthcare) and equal amounts of protein were immunoprecipitated using antibodies against total STAT3 (Santa Cruz), phospho-Y705 STAT3 (Abcam), or control IgG antibody (Abcam). Precipitates were analyzed by immunoblotting. For coIPs, p53 was immunoblotted with an HRP-conjugated p53 antibody (Santa Cruz). 5% of each input was used as input control and stained with beta-Actin (Abcam) as loading control. To stimulate STAT3, cells were treated with 50 ng/mL IL-6 or OSM 24 h prior to performing the CoIP.

Cycloheximide Chase

To evaluate the stability of different mutp53 proteins in the panel of PDAC cell lines, Cycloheximide (CHX) chase experiments were performed. Cells were treated with 40 µg/mL Cycloheximide (Sigma-Aldrich) or ethanol vehicle control for 8 h and 24 h. Protein lysates were prepared with RIPA buffer as described in immunoblot analysis.

Cell Growth Assay After Hsp90 Inhibition

To investigate HSP90 chaperone dependent stabilization of different mutp53 proteins, cells were treated with Hsp90 ATPase inhibitors Ganetespib (Synta Pharmaceuticals) or Onalespib (Selleckchem). To determine cell confluency, cells were seeded in 96-wells (Corning) and treated with Onalespib or Ganetespib for 24 h. Confluency was determined using the Celigo Imaging Cytometer and the according software (Nexcelom, Software v5.0.0.0).

Treatment With Cytokines (IL-6, OSM)

To stimulate the STAT3 pathway, cells were seeded in 6-well plates (Sarstedt) and treated with Interleukin-6 (IL-6) or Oncostatin M (OSM 209a.a.) (both from Immunotools) or solvent control for 24 h and analyzed by immunoblots.

Cell Viability Assay After Stattic Treatment

Cells were seeded in 96-well plates (Corning) and treated with increasing concentrations (0-80 µM) of Stattic or solvent control for 24 h. The CellTiter-Glo® Luminescent Cell Viability Assay (Promega), based on detectable ATP, was performed according to manufacturer's guidelines. Each biological replicate was measured in triplicates, and viability was calculated relative to the solvent control for each cell line.

Wound Healing Assay

Twenty-four hours after transfection with siRNAs or scrambled control, cells were incubated in serum-reduced media (1% FBS). Forty-eight hours post transfection, three scratches per well were made with a 1ml pipette tip or 200µl pipette tip as dublicates. Forty-eight hours after scratching, at least five images per scratch were taken, quantified, and averaged per experiment. The degree of wound healing was determined by measuring the scratched area per image using the 'polygon selection function' of Image J software. Wound healing rate was measured by averaging each scratch area after 48 h relative to the initial area at 0 h. Biological replicates are defined as independent experiments with cells at different passages and different days. For technical replicates, cells from one experiment were seeded in two different wells (duplicates).

Transwell Migration Assay

Cells were either transfected with siRNA against TP53 mRNA, STAT3 mRNA or scrambled control. Seven-two hours after siRNA transfection, cells were trypsinized and seeded into transwell inserts (Corning) in serum-reduced media (1% FBS for MIA-PACA-2, PANC-1, BXPC-3 and PA-TU-8902; 0.5% FBS for PA-TU-8988T). Wells (Corning) were filled with the respective complete medium of each cell line. To investigate migration potential upon the STAT3 inhibitor Stattic, cells were seeded in transwell inserts in serum-reduced media. Different concentrations of Stattic or respective control were added to the cells 1-2 h after seeding, allowing cells to settle before treatment. Wells were filled with complete medium. Attempting to induce migration of PA-TU-8902 cells, cells were pre-seeded in 6-well plates (Corning) and pre-treated with 50 ng/mL IL-6 or OSM (Immunotools). After 24 hrs pre-treatment, cells were transferred to transwell inserts, and cytokines were added again.

In the final 24 h after seeding, cells that had migrated to the underside of the membrane were carefully washed with PBS, fixed in ice-cold methanol for 10 min and stained with crystal violet (0.1% in 20% EtOH) for 20 min. After washing, remaining

cells inside the insert were removed using a pre-wet Q-tip. The migrated cells were visualized by light microscopy and analyzed using Image J. The migration rate was calculated relative to scrambled siRNA or solvent control, respectively. Biological replicates are defined as independent experiments with cells at different passages and different days. For technical replicates cells from one experiment were seeded in two different transwell inserts (dublicates).

Analysis of Human Patient TCGA Data

Human genomic data including *TP53* gene mutation and clinical information were downloaded from cBioPortal (www.cbioportal.org). We used cBioportal Pancreatic ductal adenocarcinoma database in this analysis (39, 40). Two datasets were used to detect mutated samples and the clinical data, QCMG, Nature 2016, and TCGA, PanCancer Atlas (41, 42). *TP53* missense mutant group was sampled with *TP53* missense mutations (MS) with indicated amino acid changes, and the *TP53* LOF group was sampled with frameshift (FS) and nonsense (NS) *TP53* mutations. R language (The R Project for Statistical Computing, https://www.r-project.org, version 4.0.2) and the package "survival" were used in the analysis, including calculating log-rank p-value and Kaplan–Meier curves.

Statistical Analysis

The number of biological and technical replicates (mean ± SEM) is provided in the figure legends. For all experiments, an unpaired Student's t test (two-sided) was used to calculate p-values.

RESULTS

p53 Missense Mutants in Human PDAC Cell Lines Are Stabilized *via* Hsp90

Since different p53 mutants have different conformations and thus different tumorigenic functions that additionally depend on specific cellular/oncogenic context, each allele and tumor type constellation should be considered separately (6, 10, 17, 43). To investigate the allele specificity of mutated *TP53* in pancreatic ductal adenocarcinoma (PDAC), we used homozygous human PDAC cell lines expressing different endogenous p53 hotspot and non-hotspot missense mutants. The panel included CAPAN-1 ($p53^{A159V}$), BXPC-3 ($p53^{Y220C}$), PANC-1 ($p53^{R273H}$), MIA-PACA-2 ($p53^{R248W}$), PA-TU-8902 ($p53^{C176S}$), and PA-TU-8988T ($p53^{R282W}$). L3.6pl harbors a truncating LOF mutation and served as p53null control. Unfortunately, an established PDAC line with a mutated $TP53^{R248Q}$ allele is not available. The absence of wildtype p53 function was verified in all cases (**Supplementary Figure 1**).

Comparative immunoblot analysis identified the highest steady state protein levels in MIA-PACA-2 cells expressing the R248W mutant (**Figure 1A**). The second highest levels were observed in C176S and R282W harboring PA-TU-8902 and PA-TU-8988T cells, respectively. The lowest level was seen in A159V expressing CAPAN-1 cells (**Figure 1A**). Cycloheximide chase experiments confirmed that the highest p53 steady state levels in cells harboring $mutp53^{R248W}$, $mutp53^{C176S}$, and $mutp53^{R282W}$ were also the most stable proteins with the longest half-lives, while mutant p53 protein with the lowest level (A159V) had the shortest half-life (**Figure 1B**).

A key prerequisite for the gain-of-function (GOF) of some missense p53 mutants is protein stabilization through the Hsp90 chaperone machinery. Importantly, the clinically relevant Hsp90 inhibitors Ganetespib or Onalespib provide therapeutic selectivity toward tumor epithelial cells but not normal cells, making them attractive for anti-cancer therapies (44). Furthermore, in other cellular contexts such as lymphoma (23), treatment with the Hsp90 inhibitor Ganetespib downregulated mutp53 protein levels. In most PDAC cells, except BXPC-3 cells (**Figure 2A**), Ganetespib or Onalespib also decreased mutp53 protein indicating that mutp53 proteins are mainly stabilized in this context by the Hsp90 chaperone machinery. In line with this, PANC-1, MIA-PACA-2, PA-TU-8902, and PA-TU-8988T cells showed diminished cell growth by about 40%, while the other lines had less reduction (**Figure 2B**). These data reinforce that at least some mutp53 proteins in PDAC might also be targetable with Hsp90 inhibitors.

The $p53^{R248W}$ Mutant Selectively Promotes Migration in PDAC Cells

We previously established that a main GOF activity of the $mutp53^{R248W}$ and $mutp53^{R248Q}$ in colorectal cancer compared to p53 null is promotion of cell migration and invasion in tumors *in vivo* and *in vitro* (26). To test whether this is also the case in PDAC, we performed migration assays. Of note, transwell migration assays showed that only siRNA-mediated depletion of $mutp53^{R248W}$ decreased the migration capacity of MIA-PACA-2 cells, while depletion of other alleles failed to do so (**Figures 3A–D**). Interestingly, PA-TU-8988T and PA-TU-8902 cells, which also express high levels of stabilized $mutp53^{R282W}$ or $mutp53^{C176S}$, respectively (**Figures 1A, B**), did not show reduced migration after mutp53 depletion (**Figure 3C**) or were completely unable to migrate through the pores of the transwell membrane (**Figure 3E**). This remained even after treatment of PA-TU-8902 cells with the cytokines Interleukin-6 (IL-6) and Oncostatin M (OSM) (**Figure 3E**), known to induce migration and proliferation in numerous cell types (45–47). This suggests that high mutp53 stabilization per se is a necessary but not sufficient precondition for acquiring a GOF on migration.

To confirm the effects seen in migration assays, three cell lines were further analyzed by wound healing scratch assays. Again, specifically MIA-PACA-2 cells bearing the R248W mutation showed the strongest reduction in wound closing capacity upon mutp53 depletion (**Figures 3F–H**).

$Mutp53^{R248W}$ Selectively Binds to Phosphorylated STAT3 in PDAC Cells

In colorectal carcinoma, an important mechanism of tumor invasion is mediated by $mutp53^{R248Q/W}$-pSTAT3 signaling by forming a physical complex (26). Reduced migration capacity of MIA-PACA-2 cells after $mutp53^{R248W}$ depletion (**Figures 3A, F**)

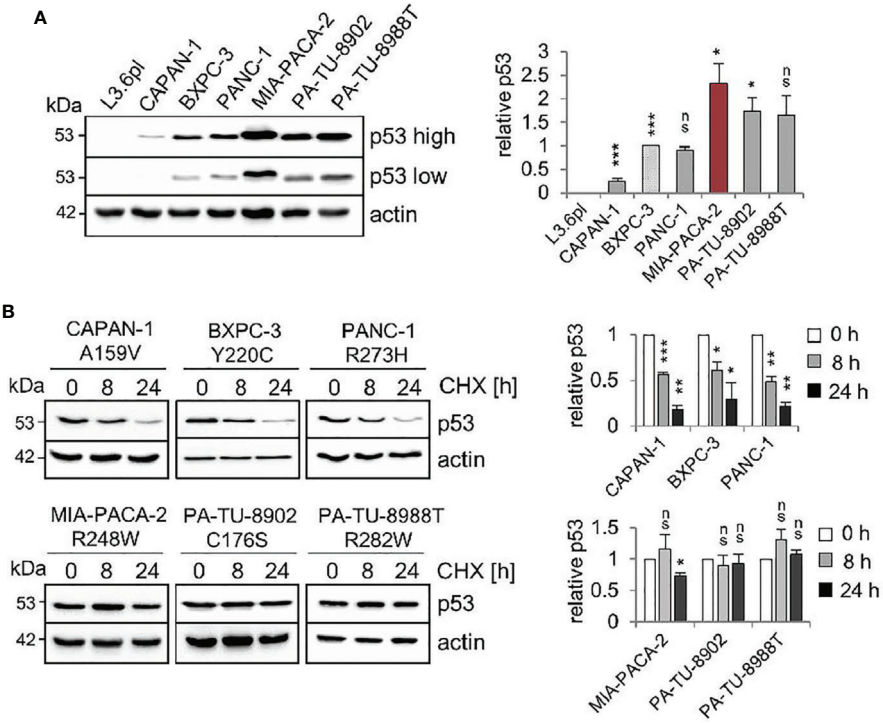

FIGURE 1 | Stabilization of various missense p53 mutants in human PDAC cell lines. **(A)** Six PDAC cell lines harboring various missense mutant p53 variants exhibit differential steady state protein levels. One representative immunoblot analysis out of four is shown. Actin as loading control. 'p53 high' and 'p53 low' mean exposure time. CAPAN-1 (mutp53^{A159V}), BXPC-3 (mutp53^{Y220C}), PANC-1 (mutp53^{R273H}), MIA-PACA-2 (mutp53^{R248W}), PA-TU-8902 (mutp53^{C176S}) and PA-TU-8988T (mutp53^{R282W}). L3.6pl cells harboring a truncating LOF mutation served as p53 null control. (right) Diagrams represent the means ± SEM of densitometric quantifications of two independent experiments with two technical replicates each (total n = 4 immunoblots), normalized to actin or HSC70 and calculated relative to mutp53 level in BXPC-3 cells (patterned bar). **(B)** Differential half-lives of mutp53 proteins. Cycloheximide (CHX) chase experiment. Cells were treated with CHX for 8 and 24 h or vehicle control (0 h). One representative immunoblot. Actin, loading control. (right) Diagrams represent mutp53 protein levels as means ± SEM of densitometric quantifications of two independent experiments (n = 2), normalized to actin or HSC70. Calculated relative to control treatment (0 h). **(A, B)** Student's t test. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ns, not significant.

suggests a similar mechanism. Since the STAT3 pathway is also an important driver of PDAC tumorigenesis (48, 49), we asked whether mutp53^{R248W}-regulated migration is similarly mediated through STAT3 signaling. The PDAC panel showed high constitutive levels of phosphorylated STAT3 (pSTAT3) in five of the seven cell lines (**Figure 4A**). Only two cell lines, PA-TU-8902 and PA-TU-8988T, had very low levels of activated STAT3 (yet exhibited significant stabilization of mutp53). On the other hand, this immunoblot analysis that examines relative ratios of both proteins indicated that four lines with high pSTAT3 had very low or undetectable mutp53 levels. Importantly, MIA-PACA-2 cells, as the only cell line with dually high levels of both mutp53 and pSTAT3, seem to fulfill the best precondition to promote migration *via* this axis.

Thus, co-immunoprecipitations (CoIPs) were performed to test which of the various mutp53 proteins are able to bind STAT3. Indeed, R248W in MIA-PACA-2 cells showed the strongest binding to total STAT3 protein compared to BXPC-3 and PANC-1 cells, forming a constitutive endogenous signaling complex (**Supplementary Figure 2A**). Since phosphorylation status is another important parameter for binding to STAT3, these cell lines with different mutp53 variants and stabilization levels were subjected to CoIPs with an antibody specific for phosphorylated STAT3. Among these mutants analyzed, mutp53^{R248W} in MIA-PACA-2 cells again showed the strongest binding to pSTAT3 (**Figure 4B**). CAPAN-1 cells with low mutp53 level showed a minor binding to pSTAT3 (**Figure 4C**) such as BXPC-3 and PANC-1 cells (**Figure 4B**) (yet exhibited moderate levels of mutp53 compared to CAPAN-1). However, PA-TU-8988T cells with intermediate mutp53 levels (lower than in MIA-PACA-2 but higher than in PANC-1 or BXPC-3 cells) again showed a strong binding of mutp53^{R282W} to pSTAT3 (**Figure 4D**). This confirms a point made in colorectal carcinoma that the ability of mutp53 to bind to pSTAT3 correlates with the degree of its stabilization (26).

To investigate if the mutp53-pSTAT3 complex can directly regulate the phosphorylation status of STAT3 as shown in CRC (26), we depleted mutp53 in MIA-PACA-2, PA-TU-8988T, PANC-1, BXPC-3 and PA-TU-8902 cells (**Figure 4E** and **Supplementary Figure 2B**). In MIA-PACA-2 and PA-TU-8988T cells, both with a strong mutp53-pSTAT3 complex formation, only mutp53^{R248W} regulated STAT3 activity in

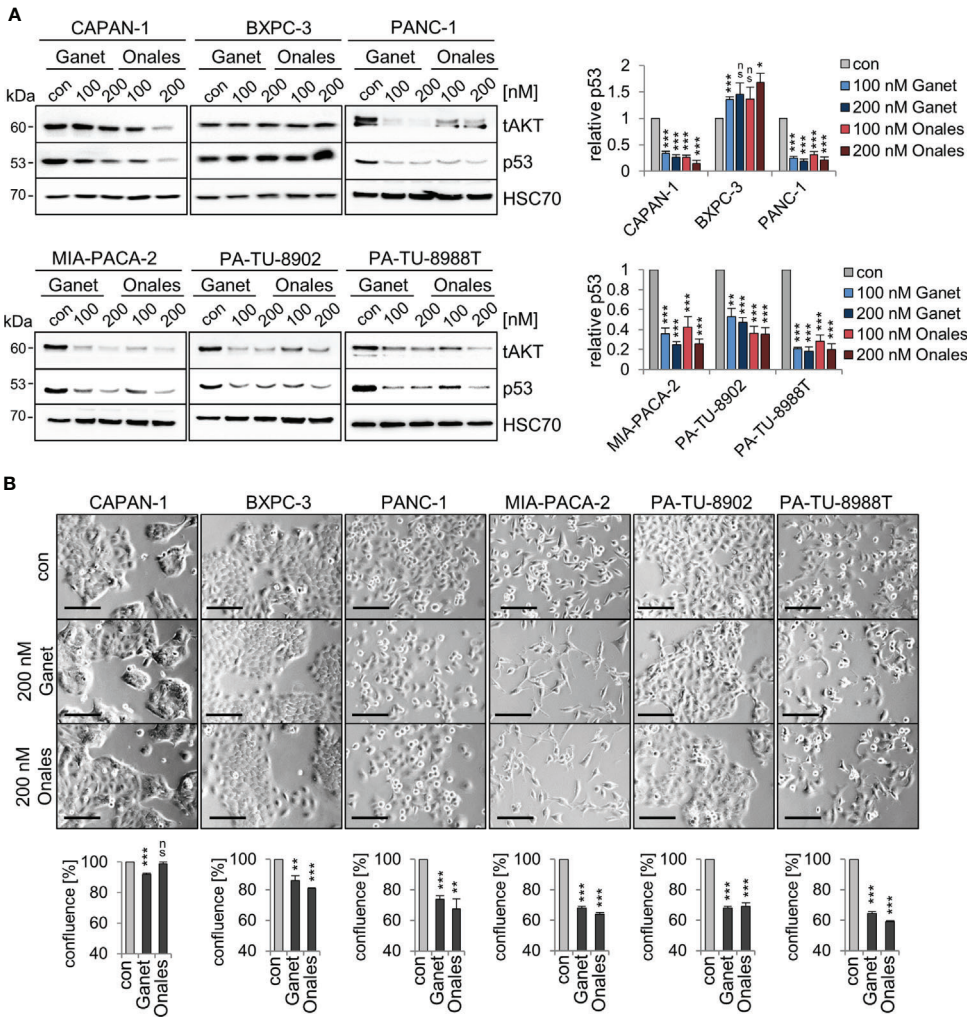

FIGURE 2 | Missense p53 mutants in PDAC cells are stabilized by Hsp90. **(A)** Hsp90-dependent aberrant stabilization of mutp53 proteins in PDAC cell lines. Cells were treated for 24 h with the indicated concentrations of Ganetespib, Onalespib, or DMSO. One representative immunoblot out of three each is presented. HSC70, loading control. Total AKT ('tAKT', AKT serine/threonine kinase 1) as well-known Hsp90 client serves as functional control for an Hsp90 inhibition. (right) Diagrams represent the means ± SEM of densitometric quantifications of at least two independent experiments with technical replicates (total n ≥ 3 immunoblots), normalized to HSC70. Calculated relative to control DMSO treatments (con). **(B)** Cell confluence determination. Representative images of cells after treatment with 200 nM Ganetespib, Onalespib, or solvent control for 24 h. Cell confluency was analyzed using a Celigo imaging cytometer. Scale bars, 100 µm. Confluence was calculated relative to their respective DMSO control from n = 3 biological replicates. **(A, B)** Student's t test. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ns, not significant.

PDAC cells, as indicated by decreased STAT3 phosphorylation selectively in MIA-PACA-2 cells (**Figure 4E**). In all other cell lines tested, pSTAT3 level were not decreased after mutp53 depletion (**Figure 4E** and **Supplementary Figure 2B**). Why mutp53 binding to pSTAT3 failed to reduce STAT3 activity in PA-TU-8988T cells remains speculative but confirms the reduced migration capacity after mutp53 depletion exclusively in MIA-PACA-2 cells (**Figures 3A–D**). These data further underline the strong invasive GOF function of the mutp53^{R248W} allele reaching across cancer entities.

Although most PDAC cell lines already exhibited high constitutive levels of pSTAT3 at baseline (**Figure 4A**), treatment with Interleukin-6 (**Figure 4F**) or Oncostatin M (**Figure 4G**) further stimulated the STAT3 pathway and induced additional increase in phosphorylated STAT3. Thus, to further evaluate whether the mutp53 binding capacity to pSTAT3 increases with higher pSTAT3 levels, MIA-PACA-2, as well as PANC-1 and BXPC-3 cells (both with a low binding capacity), were treated with IL-6, OSM, or solvent control. Interestingly, even after this strong induction of pSTAT3, the p53^{R248W} mutant showed by far the strongest binding to pSTAT3, again emphasizing allele selectivity (**Figure 4H**). These data suggest that it is not the level of pSTAT3 that predicts p53 binding in PDAC, but rather the nature of the mutp53 variant. In sum, mutp53^{R248W} shows a strong ability for complexing with pSTAT3 and regulation of migration, independent of the levels of phosphorylated STAT3.

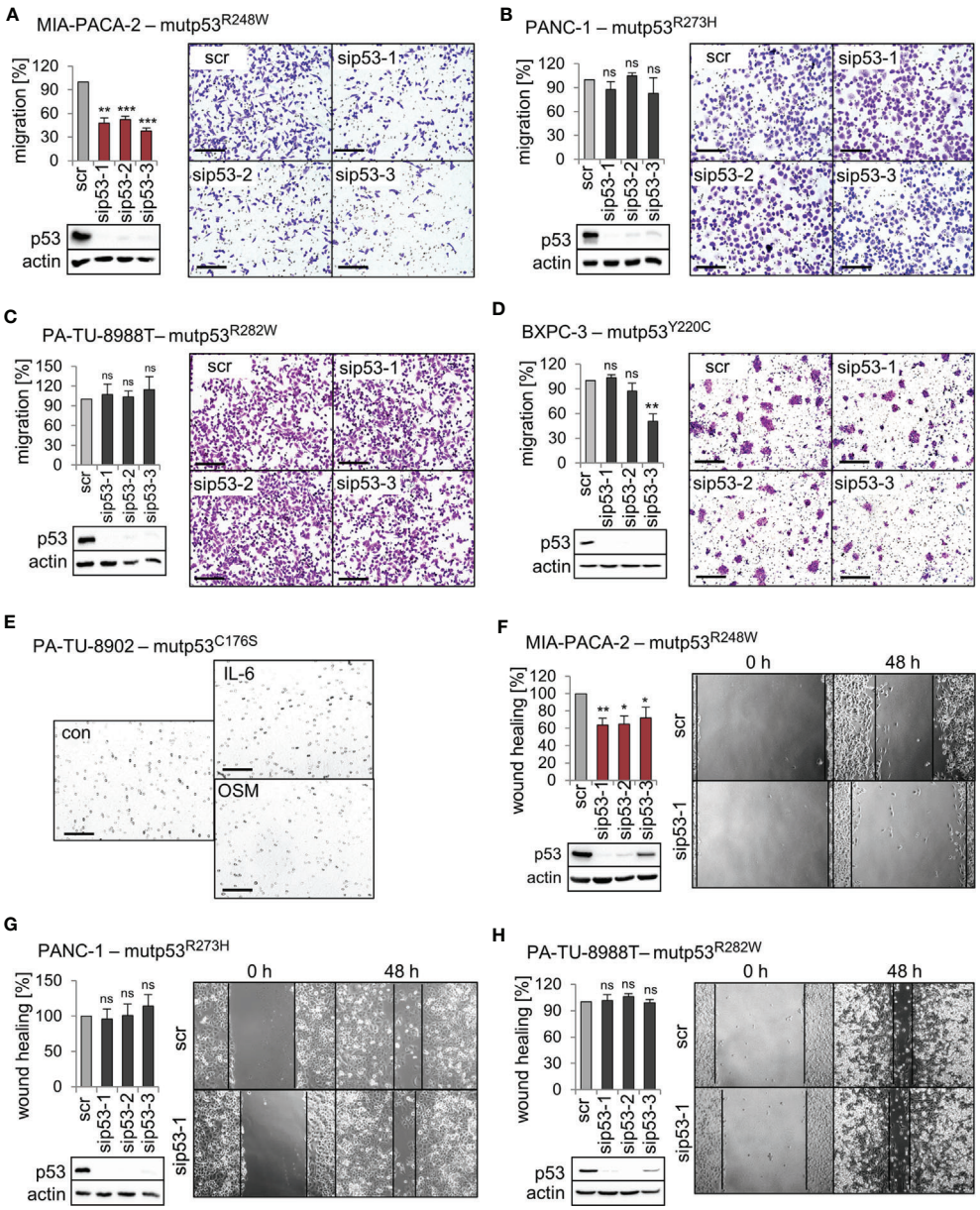

FIGURE 3 | Mutp53^{R248W} selectively promotes migration in PDAC cells. **(A–D)** Transwell migration assays of MIA-PACA-2, PANC-1, PA-TU-8988T, and BXPC-3 cells to evaluate mutp53-dependent migration activity. mutp53 was depleted with three different siRNAs against *TP53* mRNA (sip53 1-3). Seventy-two hours post-transfection with siRNAs, cells were seeded into transwell inserts and migration to the membrane underside was determined after 24 h. MIA-PACA-2 cells: 3 biological replicates (n = 3), PANC-1 cells: 2 biological replicates (n = 2), PA-TU-8988T cells: 3 biological replicates (n = 3), BXPC-3 cells: 3 biological replicates, one with a technical replicate (n = 4). Note, siRNA 'sip53-3' reduced migration in BXPC-3 cells might be a consequence of siRNA off-target effects. Migration was calculated relative to scrambled control (scr, set as 100%). Representative images of membrane undersides are shown. Scale bars, 200 µm. Immunoblot analysis verifies knockdown of mutp53. Actin, loading control. **(E)** Transwell migration assay of PA-TU-8902. Representative images of stained transwells after 24 h of migration are shown. To induce migration, cells had been stimulated for 24 h with 50 ng/mL Interleukin-6 (IL-6), Oncostatin M (OSM), or solvent control (con) prior to seeding into inserts, followed by additional cytokine treatment for another 24 h. Gray dots are pores of the membrane. Scale bars, 200 µm. **(F–H)** mutp53-dependent wound healing of MIA-PACA-2, PANC-1, and PA-TU-8988T cells. mutp53 knockdown for 48 h using three different siRNAs (sip53 1-3). Forty-eight hours post-transfection, scratch assays were performed for another 48 h. A minimum of five images were taken and quantified. MIA-PACA-2 cells: 3 biological replicates, 1 out of 3 with a technical replicate (n = 4), PANC-1 cells: 2 biological replicates, 1 out of 2 with a technical replicate (n = 3), PA-TU-8988T cells: 2 technical replicates (n = 2). Wound healing capacity was calculated relative to scrambled control (scr). Representative images after 0 h and 48 h are shown. Solid lines represent edges of the scratch. Immunoblots verify knockdown of mutp53. Actin, loading control. **(A–D, F–H)** Diagrams represent the means ± SEM. Student's t test. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ns, not significant.

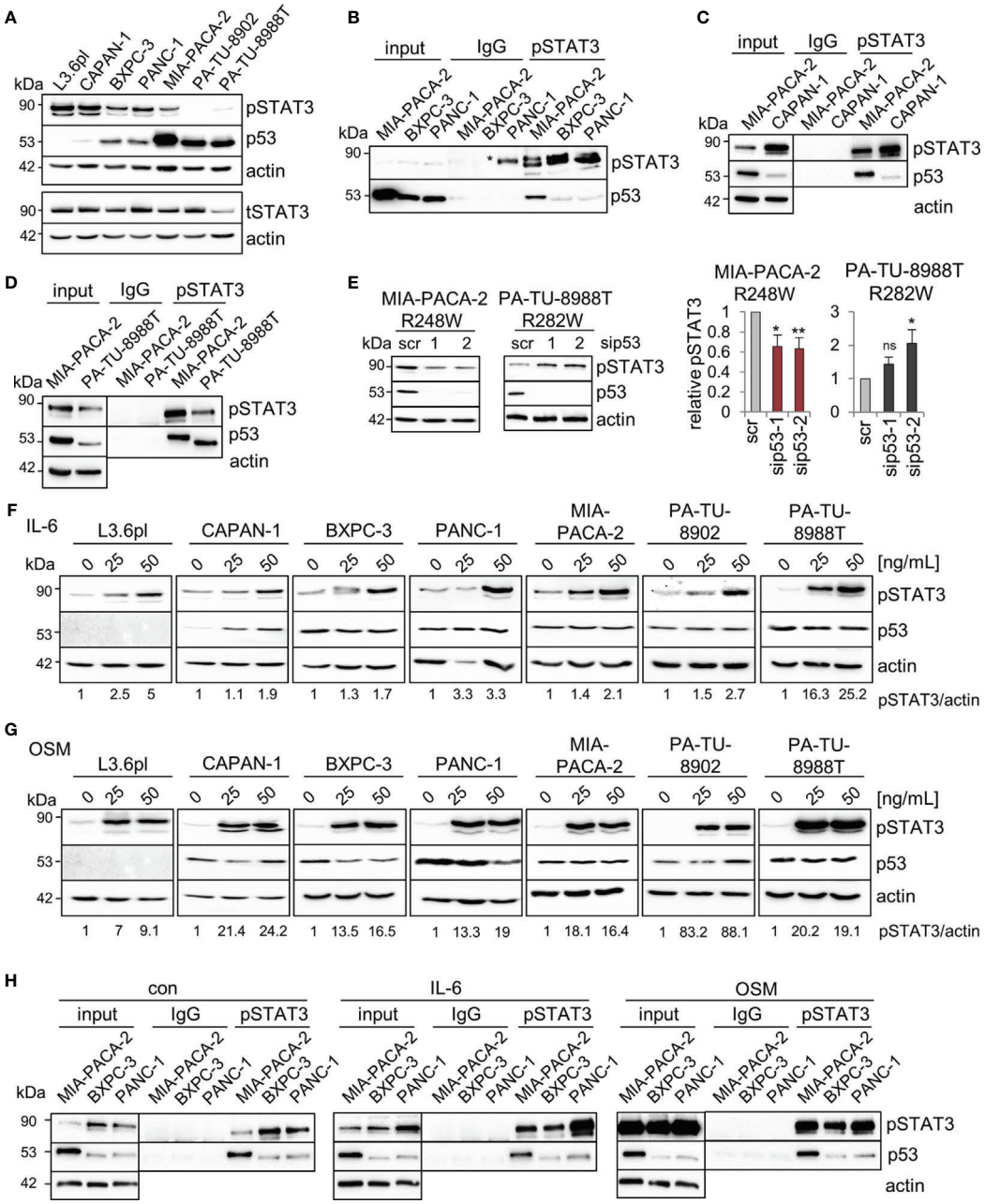

FIGURE 4 | Mutp53^{R248W} selectively binds to phosphorylated STAT3 in PDAC cells. **(A)** Representative immunoblot analysis of seven different PDAC cell lines. pSTAT3, pTyr 705-STAT3 (Y705), and total STAT3 (tSTAT3). Actin, loading control. **(B–D)** Co-immunoprecipitations (CoIPs) of untreated MIA-PACA-2, PANC-1, BXPC-3 **(B)**, CAPAN-1 **(C)**, and PA-TU-8988T **(D)** cells using anti-pSTAT3 (Y705) or IgG antibodies followed by immunoblot analysis. MIA-PACA-2 cells were always used as positive control. Note that the pSTAT3 band marked by an asterisk in **(B)** is an artefact due to a leaky pocket from the neighboring MIA-PACA-2 lane. **(E)** Knockdown of mutp53 in MIA-PACA-2, but not in PA-TU-8988T cells downregulates pSTAT3 levels. Cells were transfected with two different siRNAs against *TP53* mRNA (sip53-1, -2) or scrambled control (scr) for 72 h followed by immunoblot analysis. Representative immunoblot out of 3 (MIA-PACA-2) and out of 4 (PA-TU-8988T). Actin, loading control. (right) Diagrams represent the means ± SEM of densitometric quantifications of three (MIA-PACA-2, n=3) or two (PA-TU-8988T, n = 4) independent experiments, normalized to actin. Calculated relative to control scrambled siRNA (scr). Student's t test. *$p \leq 0.05$; **$p \leq 0.01$; ns, not significant. **(F, G)** Treatment of PDAC cell lines with the indicated concentrations of Interleukin-6 (IL-6, **F**), Oncostatin M (OSM, **G**), or respective solvent controls for 24 h. Representative immunoblot for pSTAT3 (Y705) induction is shown. Quantification by densitometry, normalized to actin loading control (pSTAT3/actin ratio) and calculated relative to solvent control. 'pSTAT3/actin', densitometric quantifications of the representative immunoblot, normalized to actin and relative to 0 ng/ml IL-6 or OSM treatments. **(H)** CoIPs of MIA-PACA-2, PANC-1 and BXPC-3 cells stimulated with 50 ng/mL IL-6, OSM or solvent control for 24 h. Immunoprecipitation using anti-pSTAT3 (Y705) or IgG antibodies, followed by immunoblot as indicated. Actin in unprecipitated input lysates, loading control. **(B–D, H)** Five percent of input were used for input control.

Mutp53^{R248W} Selectively Regulates STAT3 Phosphorylation and Activity in PDAC Cells

The above findings led us to hypothesize that mutp53^{R248W} binds to and deregulates pSTAT3 in PDAC cells by forming an oncogenic complex. Since mutp53^{R248W} depletion also selectively suppressed phosphorylation and thus activation of STAT3 (**Figure 4E**), we next asked whether the R248W mutant can be functionally linked to STAT3 dependency for migration in PDAC cells. To this end, we determined migration capacity after STAT3 ablation. Indeed, depletion of STAT3 suppressed migration ability in mutp53^{R248W} expressing MIA-PACA-2 cells (**Figure 5A**) but not in mutp53^{R273H} expressing PANC-1 cells (**Figure 5B**).

To confirm that phosphorylated STAT3 is critical for the oncogenic mechanism of the tumor-promoting mutp53^{R248W}-pSTAT3 complex, we used the small-molecule STAT3 inhibitor Stattic. Stattic selectively inhibits activation of STAT3 through interference with dimerization and nuclear translocation (50). It has been shown that Stattic substantially reduces STAT3 phosphorylation in colorectal, liver, and breast cancer cells (50–52) as well as in PDAC cells such as MIA-PACA-2 and PANC-1 (53–55). Importantly, among the panel of PDAC cells, R248W expressing MIA-PACA-2 cells were again the most susceptible to pSTAT3 inhibition by Stattic with the lowest IC_{50} value (8 μM) in cell viability assays (**Figure 5C**). Likewise, migration after Stattic treatment was strongly suppressed in MIA-PACA-2 cells (by 70%), but lower suppressed in PANC-1 (by 15%) or PA-TU-8988T cells (by 45%) cells (**Figure 5D**).

The mutp53^{R248W}-pSTAT3 complex might accelerate tumor progression in PDAC patients as we had previously seen in CRC patients (26). Indeed, patient data support this notion since PDAC patients harboring *TP53*^{R248Q} or *TP53*^{R248W} mutations showed a trend for reduced survival compared to patients with loss-of-function NS+FS mutation (**Figure 5E**), supporting the mutp53^{R248W}-pSTAT3 complex as a potentially attractive target in PDAC. Furthermore, we analyzed other missense mutants such as mutp53^{R159}, mutp53^{R175}, mutp53^{Y220}, mutp53^{R273}, and mutp53^{R282}. However, TCGA data do not provide enough PDAC cases for a sufficient statistical analysis (**Supplementary Figure 3A**). Albeit we see a tendency that other stabilized missense p53 mutants shorten patient survival, which indeed might provide attractive targets as well, more analysis is needed to explore GOF activities that are acquired by other p53 mutants (**Supplementary Figure 3B**).

In conclusion, targeting the tumor-promoting mutp53^{R248W}-pSTAT3 complex by STAT3 depletion or pharmacological inhibition diminished cell viability and migration in mutp53^{R248W} expressing, but not in mutp53^{R273H} expressing, PDAC cells.

DISCUSSION

The phenotype of p53 missense mutants is heterogenous and moreover depends on the cellular context (16–18). Here, we analyze a panel of p53 missense mutants (mutp53) in a series of homozygous human PDAC cell lines and compare the impact of various mutants on protein properties and functions. We find that mutp53^{R248W} protein undergoes strong Hsp90-mediated stabilization and selectively promotes migration by engaging in the strong constitutive complex formation with phosphorylated STAT3 at baseline and upon cytokine stimulation. Our data in pancreatic cancer suggest a R248W allele-specific gain-of-function on migration *via* STAT3 deregulation. These data mirror our previous findings in colorectal cancer (26) and further underline the necessity to investigate p53 missense mutants in a context- and allele-dependent manner (16, 19, 20).

Interestingly, PA-TU-8902 cells expressing intermediate stabilized mutp53^{C176S} showed strong STAT3 pathway stimulation by OSM or IL-6 (**Figures 4F, G**) but did not migrate at all in the transwell assay (**Figure 3E**), indicating that STAT3 fails to impact migration in these cells. Furthermore, PA-TU-8988T cells harboring intermediate levels of mutp53^{R282W} showed a strong binding to pSTAT3 but failed to regulate pSTAT3 level (indicating STAT3 activity) (**Figure 4E**) and failed to influence the migratory capacity in transwell assays as seen in mutp53^{R248W}-containing MIA-PACA-2 cells (compare **Figures 3A, C**). However, in principle, the mutp53^{R282W}-pSTAT3 complex confirms a point made in our colorectal carcinoma study that the ability of mutp53 to bind pSTAT3 correlates with the degree of its stabilization (26). The function that is acquired by the mutp53^{R282W}-pSTAT3 complex in PA-TU-8988T remains speculative. STAT3 is not just an important factor for PDAC migration (54, 56, 57) but is also involved in many other hallmarks of cancer to promote tumor progression (58, 59).

Thus, we find that different p53 mutants have different impacts on migration- and cell growth-associated STAT3 functions. Importantly, among *TP53* mutations, several other common alterations exist that drive PDAC (41). We cannot exclude that molecular PDAC subtypes influence mutp53 GOF activities. Other mutations and alteration might also contribute to migratory differences after depletion of mutp53 variants. To address this question, an isogenic cell panel with various *TP53* mutations is necessary. Since the maintenance of the *TP53* copy number is very crucial in relation to mutp53 protein stabilization, a CRISPR/Cas9-based isogenic cell panel might be most useful.

Mechanistically, the favored GOF hypothesis is that the nuclear presence of highly abundant stabilized mutp53 proteins, which have lost specific DNA binding capacity on their own, results in hijacking of (by binding to) other transcription factors and their specific cofactors, thereby building a complex network to divert and oncogenically reprogram their transcriptional activity (5, 6, 20, 24, 60–62). Regarding co-factors, it is conceivable that the mutp53 protein also adds p53-specific coactivators into this illegitimate mix, and/or that the canonical coactivator specific for the partnering transcription factor might get displaced. Thus, interplay networks of mutp53 with co-regulation of various tumor drivers is essential for GOF-mediated cancer progression (4, 6, 24, 60, 63). This concept could explain why the mutp53 status or the status of STAT3 phosphorylation alone is not yet a determinant for migration but depends on the specific missense mutation, resulting in specific mutp53-pSTAT3 complexes with mutp53 variant-specific transcriptional cofactors. In line with this, it is shown that mutp53^{R273H} and mutp53^{R175H} can regulate NF-κB activity in

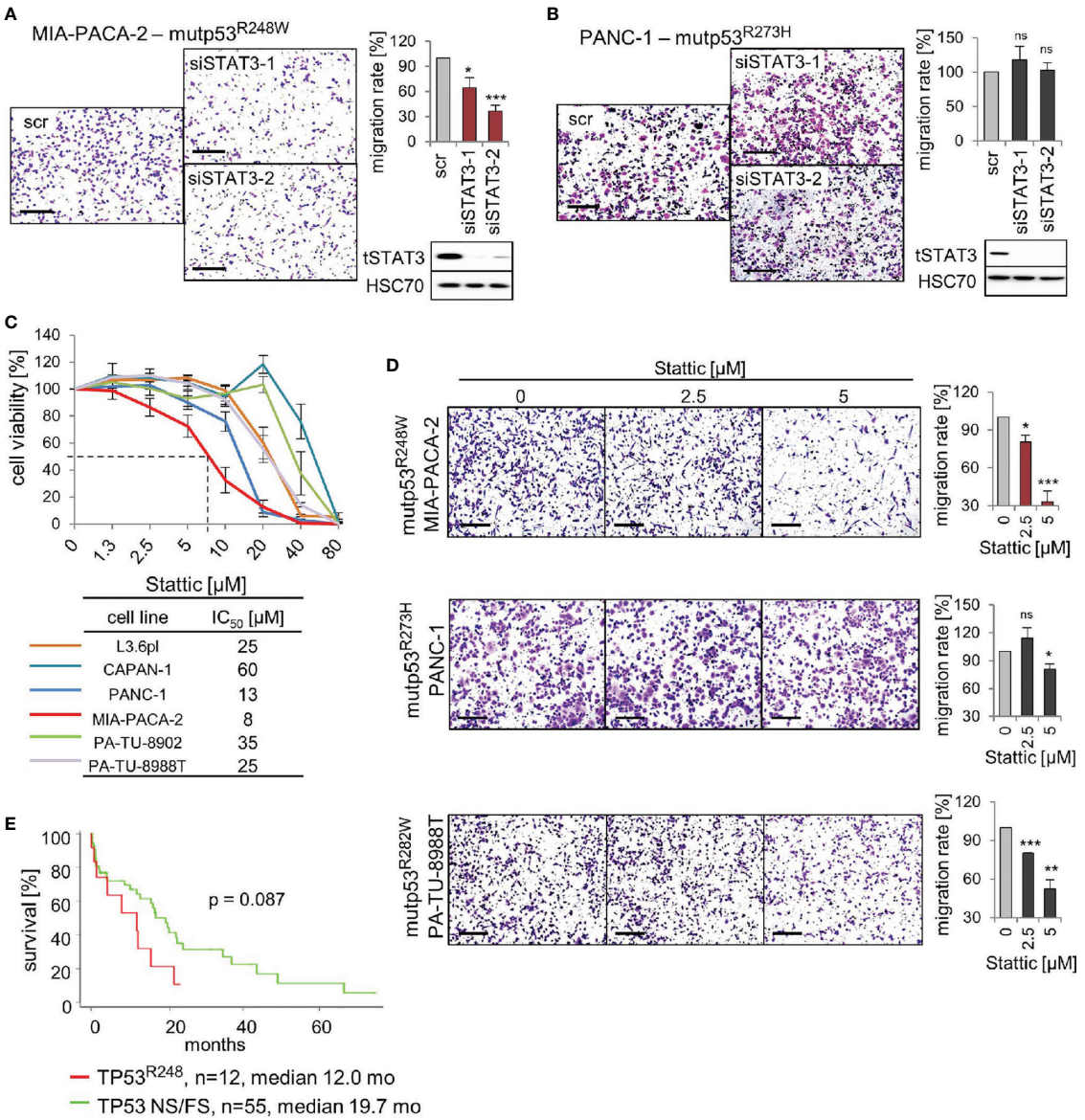

FIGURE 5 | p53^{R248W} mutant selectively regulates STAT3 phosphorylation and activity in PDAC cells. **(A, B)** STAT3 knockdown phenocopies mutp53 knockdown in migration assays. MIA-PACA-2 **(A)** and PANC-1 **(B)** cells were transfected with two different siRNAs against *STAT3* mRNA (siSTAT3-1, -2) or scrambled control (scr). Seventy-two-hour post-transfection cells were seeded into transwell inserts to assess their migration. After 24 h, cells were fixed, stained, and counted at the membrane underside. Scale bars, 200 µm. MIA-PACA-2 cells: 4 biological replicates (n = 4), PANC-1 cells: 3 biological replicates, 2 out of 3 with 2 technical replicates (n = 5). Cells were calculated relative to scrambled control. Immunoblot analysis to confirm knockdown of STAT3. HSC70, loading control. **(C)** Cell viability assays of the indicated PDAC cell lines. Dose response curve after treatment with increasing concentrations of the STAT3 inhibitor Stattic or solvent control for 24 h. For each cell line, three to four biological replicates were measured. Diagram represents means ± SEM. From these curves, IC_{50} values were determined, indicated in the table. Of note, MIA-PACA-2 cells are the most sensitive to Stattic treatment, indicated by the dashed line. **(D)** STAT3 inhibition phenocopies mutp53 knockdown in migration assays. Transwell migration assays of MIA-PACA-2, PANC-1, and PA-TU-8988T cells treated with the indicated concentrations of Stattic for 24 h. Scale bars, 200 µm. For all cell lines, quantification of two biological replicates, one of them with two technical replicates (n = 3 total), calculated relative to 0 µM control treatment. **(E)** Survival curve of PDAC patients harboring TP53 R248 mutations versus patients harboring TP53 nonsense or frameshift (NS/FS) mutations. Number of patients and mean overall survival in months as indicated. TCGA data. Kaplan–Meier statistic, log-rank test. **(A, B, D)** Diagrams represent the means ± SEM. Student's t test. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ns, not significant.

cancer cells (64, 65). Interestingly, NF-κB and STAT3 also physically interact and coregulate transcriptional pathways in cancer (66, 67). Together with our finding that mutp53^{R273H} does not significantly bind to pSTAT3 in PANC-1 cells (**Figure 4B**) and does not regulate their migration (**Figures 3B, G**), it further emphasizes the allele specificity of oncogenic mechanisms. Other studies also show context-dependent mutp53 specificities (6, 17). One example is mutp53^{R175H}, which promotes aberrant self-renewal in leukemic

cells through binding to FOXH1 as critical regulator of stem cell–associated genes (68). Furthermore, mutp53^{R175H} or mutp53$^{R273H/C}$ form complexes with NF-Y and p300 proteins to override cellular failsafe programs, thus permitting tumor progression (69). Mutp53 promotes invasion, e.g., *via* constitutive activation of EGFR/integrin signaling (70) and by antagonizing TAp63 (71).

Mutp53 stabilization occurs *via* binding to Hsp90 (5, 23), which offers therapeutic approaches to target stabilized GOF mutp53 protein in cancer cells *via* Hsp90 inhibition. Thus, treatment with the Hsp90 inhibitors Ganetespib and Onalespib diminished mutp53 levels in most analyzed PDAC cells (**Figure 2A**). However, in BXPC-3 cells, both Hsp90 inhibitors failed to destabilize Hsp90 clients (also see *Functional Control AKT*). The reason why remains speculative but resistance mechanisms are known such as an UGT1A (UDP glucuronosyltransferase 1A) overexpression (72). Importantly, in cells with a strong stabilization of mutp53 (MIA-PACA-2, PA-TU-8902, and PA-TU-8988T, **Figure 1B**), inhibition of Hsp90 resulted in significant suppression of cell growth (**Figure 2B**). In CAPAN-1 cells with a low degree of mutp53 stabilization (**Figures 1A, B**), Hsp90 inhibition did not substantially impact cell confluency (**Figure 2B**).

In sum, our preliminary *in vitro* results support a GOF of mutp53^{R248W} in pancreatic cancer, justifying future *in vivo* investigations on stabilized mutp53 as a putative therapeutic target in this important tumor entity that is in dire need of new therapeutic concepts.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Conceptualization: RS-H, LK. Methodology: RS-H, LK, CF, SS. Experimentation: LK, CF, NW, FT. Writing Original Draft LK, RS-H, UM. Writing Review and Editing: all authors. Funding Acquisition: RS-H, UM. Supervision: RS-H. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.642603/full#supplementary-material

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