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Editors' showcase insights in cell growth and division 2021

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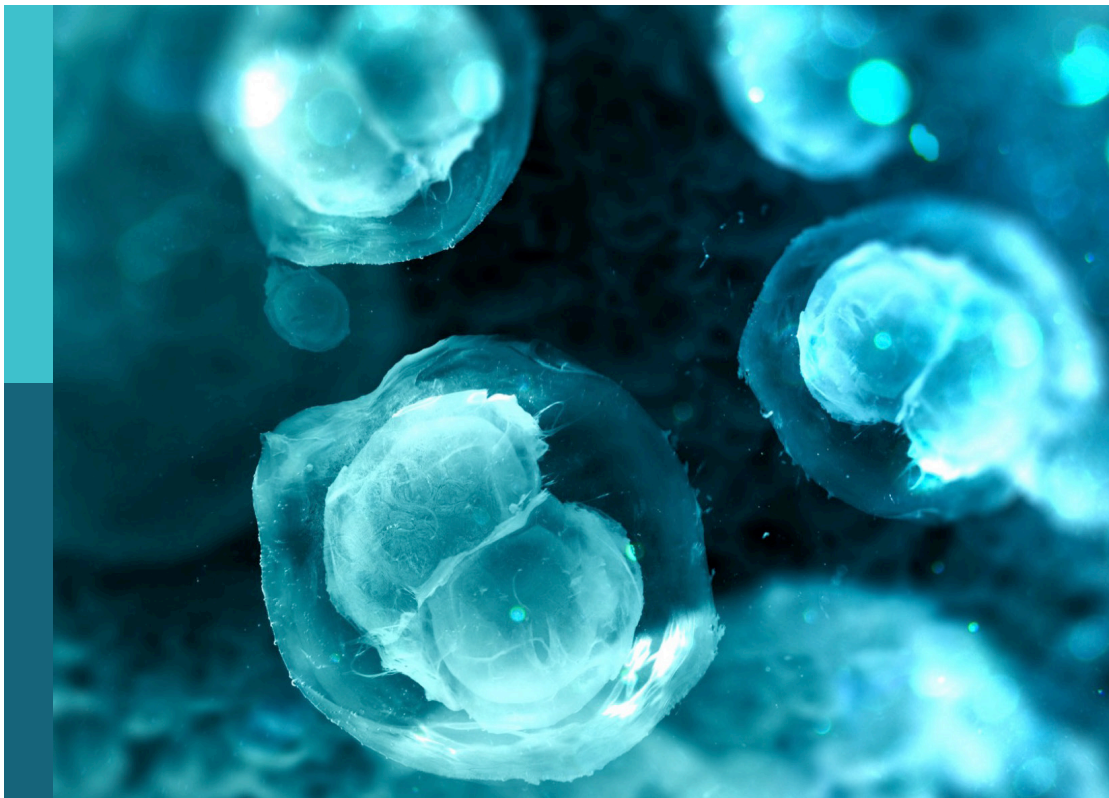

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Editors' showcase 2021: Insights in cell growth and division

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Editorial: Editors' showcase 2021: Insights in cell growth and division

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KEYWORDS

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Editorial on the Research Topic
Editors' showcase 2021: Insights in cell growth and division

The Research Topic "Insights in Cell Growth and Division" is a collection of articles published in the Cell Growth and Division section of Frontiers in Cell and Developmental Biology (https://www.frontiersin.org/journals/cell-and-developmental-biology). Since this journal views the topic of cell and developmental biology quite broadly, it only makes sense that the articles cover a lot of ground. This includes ovarian primordial-to primary follicle transition, DNA damage checkpoint, neural stem cell cycle regulators, asymmetric cell division, the mammary gland, protein degradation, functions of centrosomes, tumor suppressors, centrioles, and genome and chromosome stability (for more detail see below). While, several articles deal with cell cycle in the broadest sense, others focus on development.

For example, Thompson et at. discuss the functions of SKP1, a component of the SCF (SKP1, Cullin 1, F-box protein) complex in genome and chromosome stability and how it relates to cancer. Following a similar path, Guerber et al. present an overview of the Ubiquitin Binding Protein 2-Like (UBAP2L), which plays several roles in cancer including promoting cell proliferation, growth, EMT, migration, invasion, metastasis, vascularization, and survival.

Centrioles form centrosomes and are important components of cells, which is the topic of two reviews. Langlois-Lemay and D'Amours discuss the functions of centrosomes beyond their traditional role of microtubule organizing centers touching on cell cycle progression, DNA damage, sensory reception, and cell homeostasis. Avidor-Reiss et al. investigate the functions of centrioles in male fertility in a variety of mammals. Continuing with the theme of fertility, Ataei-Nazari et al. studied the functions of CDK6, a well-known cell cycle regulator, in oocyte development and define a new role for CDK6 in the primordial-to-primary transition. Lee reviews the mammary gland in regard to shared pathways in embryonic mammary gland cells and breast cancer.

There are three articles related to cell cycle regulation including the DNA damage checkpoint, asymmetric cell division, and neural stem cell cycle regulators. Yam et al. describe a DNA damage checkpoint at the G2/M transition with regards how it engages and how it is switched off. Mistakes in this checkpoint leads to adaptation where the cells continue to divide despite the damage. Li et al. discuss asymmetric cell division which plays an important role in stem cells. They focus on cancer stem cells and whether asymmetric cell division contributes to tumor heterogeneity and cancer progression. Caron et al. review neural stem cell cycle regulators in zebrafish. Zebrafish is an excellent model system to study

neurogenesis and neuroregeneration which identified niche-specific cell cycle behavior and novel cell cycle regulators.

Finally, Bourouh and Marignani cover the Liver kinase B1 (LKB1), an important regulator of metabolism and cancer. They focus on lung cancer and the aberrant metabolic pathways connected to LKB1 loss.

Overall, this Research Topic contains well-written articles by experts in their field that will be of great interest to a broad audience. At the same time, it shall remind us that cancer is primarily a signaling disease as was aptly described by Yaffe (2019).

Author contributions

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

Reference

Yaffe, M. B. (2019). Why geneticists stole cancer research even though cancer is primarily a signaling disease. *Sci. Signal* 12, aaw3483. doi:10.1126/scisignal.aaw3483

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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The Centriole's Role in Miscarriages

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Centrioles are subcellular organelles essential for normal cell function and development; they form the cell's centrosome (a major cytoplasmic microtubule organization center) and cilium (a sensory and motile hair-like cellular extension). Centrioles with evolutionarily conserved characteristics are found in most animal cell types but are absent in egg cells and exhibit unexpectedly high structural, compositional, and functional diversity in sperm cells. As a result, the centriole's precise role in fertility and early embryo development is unclear. The centrioles are found in the spermatozoan neck, a strategic location connecting two central functional units: the tail, which propels the sperm to the egg and the head, which holds the paternal genetic material. The spermatozoan neck is an ideal site for evolutionary innovation as it can control tail movement pre-fertilization and the male pronucleus' behavior post-fertilization. We propose that human, bovine, and most other mammals–which exhibit ancestral centriole-dependent reproduction and two spermatozoan centrioles, where one canonical centriole is maintained, and one atypical centriole is formed–adapted extensive species-specific centriolar features. As a result, these centrioles have a high post-fertilization malfunction rate, resulting in aneuploidy, and miscarriages. In contrast, house mice evolved centriole-independent reproduction, losing the spermatozoan centrioles and overcoming a mechanism that causes miscarriages.

Keywords: centriole, centrosome, sperm, miscarriage, fertility

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MAIN TEXT

Introduction

Miscarriage is a complex disease involving multiple factors in the sperm, egg, embryo, and uterus (Carbonnel et al., 2021; Klimczak et al., 2021; Thomas et al., 2021; Brandt et al., 2022; So et al., 2022). Here, we focus on the sperm centriole, a factor that has reemerged in the field of reproductive biology as a critical factor post-fertilization. Centrioles were discovered by studying reproduction in the late 19th century; still, the centriole's precise role in reproduction has proven to be a mystery (Scheer, 2014; Avidor-Reiss et al., 2020). It became evident early on that many dividing cell types require exactly two centrioles per cell, and having less or more results in abnormal or dead daughter cells (Delattre and Gönczy, 2004). The centriole's classical role is nucleating the centrosome, which acts as the dominant microtubule organization center and was characterized during the 20th century (Azimzadeh et al., 2004). However, their role remained enigmatic, as centrioles were absent in many eukaryotic life forms, such as higher plants (Wheatley, 1982; Marshall, 2009; Carvalho-Santos et al., 2010; Hodges et al., 2010). Additionally, centrosome-like structures that organize cytoplasmic microtubules can form in the absence of centrioles, as seen in yeast spindle pole bodies (SPBs), and amoebozoan nucleus-associated bodies (NABs) (Gräf, 2018). Not until the turn of the 20th century

did it become evident that most of our body's cells have an essential primary cilium (Rosenbaum and Witman, 2002). Cilium nucleation represents the ancestral role of centrioles, which requires the centriole to have its distinctive design, as it serves a structural template for the cilium axoneme (Avidor-Reiss, 2010; Avidor-Reiss et al., 2012). Only at the beginning of the 21st century were the molecular mechanisms of centriole duplication and their composition uncovered (Guichard et al., 2018; Winey and O'Toole, 2014), meaning that centrioles were the last classical sub-cellular structures with an unknown molecular assembly mechanism. We are now beginning to develop a detailed understanding of the function of the centriole's individual components, though the precise role of centrioles in sperm, and early embryo development remains perplexing.

In this perspective, we will provide insight into the elusive reproductive functions of centrioles and offer a potential explanation as to why mice evolved centriole-independent reproduction. It is important to note that many other mechanisms function in the zygote and play a critical role in assuring normal embryonic development (Bury et al., 2016; Masset et al., 2021; Mitchell, 2022). Furthermore, in addition to the centrioles, there are many other differences between mice and humans during early embryogenesis, including the timing of embryonic genome activation (Niakan et al., 2012), and the role of the master regulator of cell pluripotency (Daigneault et al., 2018).

Dividing Cells Have Precisely Two Distinct Centrioles With Evolutionarily Conserved Design

Centrioles are evolutionarily conserved organelles with characteristic structure, composition, and function in animal cells (Marshall, 2009; Carvalho-Santos et al., 2010; Hodges et al., 2010). A cell typically has two distinct centrioles: a younger and an older centriole. The younger centriole is formed in the immediately preceding cell cycle and the older centriole during past cycles (Avidor-Reiss and Gopalakrishnan, 2013; Sullenberger et al., 2020). This difference in age is coupled with distinctly different compositions and functions between the two centrioles. The older centriole is structurally mature and is therefore known as the mother centriole. It extends to form the axoneme, producing the cell's cilium in G1 or G0 of the cell cycle. The mother centriole recruits pericentriolar proteins to form the centrosome in preparation for cell division during the G2 and M phases of the cell cycle. The younger centriole is structurally immature and is referred to as the daughter centriole. Though it is linked to the mother centriole, its role in G1 of the cell cycle is unclear. However, once a cell commits to cell division, like the mother centriole, the daughter centriole duplicates in S phase, and forms a procentriole. After the subsequent M phase, the daughter centriole matures to become the mother centriole of one of the daughter cells generated during cell division. Therefore, to maintain this cycle of centriole duplication, dividing cells require two centrioles during normal animal development and physiology, and centriole formation defects result in embryo death or abnormal offspring.

Sperm Have Two Centrioles, but the Egg Does Not

Like other cell types, early mammalian egg cells (pre-pubertal oocytes) have two centrioles, but they degenerate, and disappear during egg maturation (Simerly et al., 2018). Ultimately, meiotic cell division is mediated without recognizable centrioles (Manandhar et al., 2005; Simerly et al., 2018). The fate of the centrioles is different in sperm.

Early sperm cells (spermatids) also have two centrioles, named based on their location. The proximal centriole (PC) is located near the sperm nucleus and forms a transient, cytoplasmic, axoneme-like extension known as the centriolar adjunct (Garanina et al., 2019). The second centriole, the distal centriole (DC), is located farther away from the nucleus, and forms the sperm tail axoneme. In this centriole pair, the PC is likely the mother centriole, while the DC is the daughter centriole (Grier, 1973; Alieva et al., 2018).

For a long time, it was thought that human sperm provided one functional centriole (the PC), one degraded or remnant centriole (the DC), and no pericentriolar material (PCM) to the egg (Manandhar et al., 2005; Luo et al., 2013). However, recently it became evident that human, bovine, and probably most other mammalian sperm have two functional centrioles and PCM, though they may exhibit atypical designs (Fishman et al., 2018; Amargant et al., 2021; Khanal et al., 2021; Leung et al., 2021). The PC displays slightly modified structure and composition, while the DC displays highly modified structure and composition. The usually amorphous PCM becomes the highly structured striated columns (SCs) and capitulum in the spermatozoon. The PC and DC (possibly with the SCs) form the zygotic centrosomes.

The Two Sperm Centrioles Form Two Essential Zygotic Centrosomes in Humans and Most Animals

For a long time, it was unclear how the mammalian zygote acquired its first two centrosomes, as it was thought that sperm provided only the PC (Schatten and Sun, 2009; Avidor-Reiss et al., 2015). However, it recently became evident that spermatozoa have a second, atypical centriole, the DC. Shortly after fertilization, the two spermatozoan centrioles stay together at the base of the decondensing male pronucleus, recruiting egg PCM proteins, and forming the first zygotic centrosome, which emanates a large aster (aka, the sperm aster). Later, the PC and DC (which is still attached to the axoneme) separate to form two independent centrosomes that are first located at the junction of the male and female pronuclei and, later, at the pole periphery of the male and female parallel spindles (Fishman et al., 2018; Cavazza et al., 2021; Kai et al., 2021; Schneider et al., 2021). The two sperm centrioles appear to be essential post-fertilization, as it is impossible to achieve live birth by fertilizing the egg with only sperm heads in humans and most other mammal species (with the notorious exception of mice and other murine species–see below) (Moomjy et al., 1999; Avidor-Reiss et al., 2019). Spermatozoan and embryonic centrioles are essential in

most studied invertebrates (nematodes and fruit flies) and base vertebrates (zebrafish), suggesting that centriole-dependent reproduction is the ancestral case (Avidor-Reiss, 2018; Blachon et al., 2014; Yabe et al., 2007; O'Connell et al., 2001).

Mice Have No Centrioles in Mature Sperm or the Early Embryo

The fate of the spermatozoan centrioles during sperm differentiation (spermiogenesis in the testes) and maturation (in the epididymis) is species-specific. Unlike most other mammals, in the house mouse (*Mus musculus*), rat (*Rattus norvegicus*), and Mongolian gerbil (*Meriones unguiculatus*), members of the Murinae family, the two mature spermatozoan centrioles are undetected (Woolley and Fawcett, 1973; Chakraborty, 1979; Manandhar et al., 1998; Simerly et al., 2016; Avidor-Reiss and Fishman, 2019). Furthermore, they, or whatever remains of them, are dispensable for early embryo development, as healthy mice and phylogenetically close species (e.g., hamsters) can be born following the injection of a tailless sperm head or nucleus into the egg (Kuretake et al., 1996; Yamauchi et al., 2002; Yan et al., 2008). Also, no centriole is detected in the embryo up to the blastula stage in natural mouse reproduction (Gueth-Hallonet et al., 1993; Coelho et al., 2013; Bangs et al., 2015). Instead of round, centriole-nucleated centrosomes, after fertilization, many small acentriolar microtubule organization centers appear in the cytoplasm, and they organize the two, unique, parallel maternal and paternal spindles (Reichmann et al., 2018). This even occurs following polyspermy (multiple paternal contribution) or parthenogenesis (no paternal contribution) (Schatten et al., 1991; Courtois et al., 2012; Zenker et al., 2017). This shows that mice exhibit novel, centriole-independent reproduction.

The Essential Role of the Sperm Centriole is Controversial

The essential role of the sperm centriole in humans and other mammals is controversial for three main reasons.

First, a common argument against the essential role of the sperm centriole in humans is that they are dispensable in mice. However, the loss of a critical biological feature in one species does not necessarily indicate the lack of significance in other animals. While the previous statement may be trivial, it is a critical point to make. To elaborate, snakes do not have legs, but legs are essential in other animals. Snakes have lost their legs through the evolution of an alternative mode of locomotion that does not rely on legs (Jayne, 2020). Yet, humans and other land animals retained the need for legs as a means of transportation (though humans evolved walking on two legs, while most other mammals maintained the ancestral mode of walking on four legs). This leg/centriole analogy raises questions as to how and why mice have evolved centriole-independent reproduction and as to what new mechanism is substituted for the performance of centriolar function. It is important to note that "mice are not humans," and they can differ in very basic subcellular processes (Fischer, 2021).

The second reason for the controversy is that it has not been demonstrated conclusively that sperm centrioles are essential post-fertilization by eliminating their function without causing significant defects in the sperm or zygote. To accomplish this, we need to eliminate the sperm centrioles without affecting other spermatozoan structures in a model system that has sperm centrioles (i.e., not in mice or rats); this requires experimentation on non-traditional model systems. Alternatively, this could be accomplished if efficient *in vitro* spermatogenesis could be achieved in humans or other models with sperm centrioles.

Finally, in the absence of sperm centrioles, an activated egg can be stimulated to form a parthenote (an embryo developed from an unfertilized egg) (Balakier and Casper, 1991). However, the mammalian parthenote does not reach live birth and usually dies by the blastocyst stage (Wininger, 2004; Paffoni et al., 2007; Amargant et al., 2021). Parthenotes that undergo division eventually gain centrioles through the process of *de novo* centriole synthesis. However, this process is highly erroneous, and parthenogenic cells (which do not inherit sperm centrioles) often have abnormal centriole numbers (Brevini et al., 2012). The presence of too many or too few centrioles can lead to chromosomal instability, cell death, and, ultimately, embryo developmental arrest (Bhak et al., 2006; Sir et al., 2013; Godinho et al., 2014). Additional comparative studies of both non-murine and mouse parthenogenic centrioles are necessary to determine the stage at which centrioles form, the number formed, and their function.

Research into this controversy has revealed at least two mechanisms that can organize the microtubule cytoskeleton in humans and most other mammals. One is a dominant mechanism mediated by the centriole/centrosome. The dominance of this mechanism is apparent in polyspermic zygotes, which are forced into multipolar spindle assembly due to the presence of a higher-than-normal number of centrosomes (Kai et al., 2021). The second is a complementary/compensatory mechanism mediated by the chromosomes and a set of regulatory and motor proteins. This process is beyond the scope of this manuscript, and more information about it can be found in (Mogessie et al., 2018; Geisterfer et al., 2021; Kiyomitsu and Boerner, 2021). Therefore, it is possible that non-murine zygotes, unlike mouse zygotes, require two centrioles to produce a viable embryo because they are more dependent on centriole-based mechanisms.

Human and Bovine Have a Naturally High Miscarriage Rate

One potential explanation for the disappearance of centrioles in mouse reproduction is that their presence may promote early embryo aneuploidy and miscarriages. In human and bovine, reproduction is associated with a high rate of defective cell division in the early embryo (**Figure 1A**). For example, multinucleated blastomeres were present in 43–44% of human embryos at the two-cell stage (Balakier and Cadesky, 1997; Aguilar et al., 2016). Also, bovine embryos have a significant miscarriage rate of up to 48% in beef cattle (Reese et al., 2020) and

pregnancy loss rates of ~60% in dairy cattle (Santos et al., 2004). Three recent studies suggest that these early embryonic multinucleations and miscarriages may be due to zygotic centrosome dysfunction.

Kai et al., (2021), using live imaging of human tri-pronuclear zygotes, found that the first mitotic spindle formation is led by sperm centrosome-dependent microtubule-organizing centers and that sperm centrioles may cause a high incidence of zygotic division errors (Kai et al., 2021).

Cavazza et al., (2021) found that two zygotic centrosomes reside at the junction of the male and female pronuclei and cluster the parental genomes in human and bovine zygotes (Cavazza et al., 2021). Defects in centrosome location or function lead to aneuploidy, abnormal chromosome segregation, the appearance of micronuclei, and impaired embryo development. This demonstrates that zygotic centrosome function is critical for embryonic development in human and bovine.

Amargant et al. (2021) estimated that the human sperm carries 251 centrosomal proteins within its tail to the egg and found that one of these proteins (pericentrin) remains anchored to the sperm centrioles in the zygote (Amargant et al., 2021). This study also showed that injection of human sperm tails into human oocytes followed by parthenogenetic activation results in more robust early cell divisions and improves the parthenote's development. They found that tail-injected parthenotes maintain better control of centriole numbers in their cells. This study demonstrates that the sperm tail and likely its centrioles contribute to embryo development.

Additional similarities identified between bovine and human early embryogenesis are absent in mice (Carreiro et al., 2021). Recently, it was reported that horses, whose sperm also have two centrioles, suffer from a high rate of aneuploidy in naturally occurring pregnancies (Shilton et al., 2020).

Together, these recent studies point to a critical role of centrosome function in the embryo that is often jeopardized, resulting in a reduction in reproductive efficiency.

Unlike Human and Bovine, Mice Have a Naturally Low Miscarriage Rate

House mice have a very low rate of mosaic aneuploidy in preimplantation embryos (about 25% of embryos, compared to 70% in humans) (Lightfoot et al., 2006), which plays a role in their negligible rate of naturally conceived miscarriages of less than 1% (Li et al., 2011; Yang et al., 2019; Sandalinas et al., 2001; Rubio et al., 2007). Also, in contrast to the much higher rate observed in humans, the spontaneous occurrence of aneuploidy in mice is rare, affecting less than 1% of embryos (Lightfoot et al., 2006; Bond et al., 1983). For example, resorbed embryos were rarely observed in wild-type females at 11.5 days postcoitus (**Figure 1B**), and 0 of 90 one-cell zygotes displayed chromosomal aberrations (Yuan et al., 2002). Only 8% of mouse IVF embryos (n = 36) failed to develop into morphologically normal blastocysts, and only 9.7% of blastomeres had significant chromosome segregation errors, such as lagging chromosomes (n = 72) (Bolton et al., 2016). Therefore, it is possible that mice evolved centriole-independent reproduction to improve early embryonic development efficiency. Centrioles appear in mice just before the embryo needs to form a cilium to support essential developmental processes, such as determination of the embryo's left-right axis. It is important to note that the house mice used in laboratories are highly inbred, multiparous mammals, compared to human and some bovine, which are outbred mammals that typically give birth to single offspring. Therefore, it would be important to study miscarriages in wild mice populations and other multiparous species with paternal sperm centriolar inheritance, such as rabbits (Vandenbergh, 2000).

Atypical Sperm Centrioles Evolved Independently in Many Animal Groups, Suggesting Convergent Evolution

Centrioles with atypical structures evolved independently in multiple animal clades, including insects, mammals, and certain fish.

In insects, the DC varies from having 20–50 singlet tubules in the fungus gnat *Sciara coprophila* (Phillips, 1967), to having nine collapsed triplet-microtubules in *Drosophila melanogaster* (Khire et al., 2016), to having nine doublet-microtubules in *Tribolium* (Fishman et al., 2017). Discovered only recently, the PC looks alien in insects. In some insects, like *Drosophila,* the PC has no microtubules at all (Blachon et al., 2009; Gottardo et al., 2015; Khire et al., 2015; Dallai et al., 2017; Fishman et al., 2017; Uzbekov et al., 2018). Still, the *Drosophila* zygote inherits both sperm centrioles (Blachon et al., 2014). These centrioles recruit PCM, form a centrosome that emanates astral microtubules, duplicate to create new centrioles, and localize to the spindle pole (Khire et al., 2016).

In non-murine mammals, such as bovine, the PC maintains the canonical centriolar structure with some modifications (Sutovsky and Schatten, 2000; Leung et al., 2021), like the loss of canonical centriolar proteins, such as Cep295 and RTTN (Fishman et al., 2018). The PC is asymmetric, with triplet microtubules of unequal lengths (Leung et al., 2021). In these mammals, the DC is highly modified and was thought to be degraded (Manandhar et al., 2000). Recently, it was shown to be made of splayed microtubules that associate with novel rod and bar structures (Fishman et al., 2017; Khanal et al., 2021; Leung et al., 2021). Still, the two sperm centrioles are inherited by the bovine zygote, where they recruit PCM, form a centrosome that emanates astral microtubules, duplicate to form new centrioles, and localize to the junction between the male and female pronuclei and later to the vicinity of the zygote's spindle poles.

The DC is canonical in six distantly related fish clades, but the PC is either atypical or undetected (Turner et al., 2017). While it is possible that these species lost their PC, another, more likely explanation is that the PC is modified to the point of being unrecognizable. This alternative explanation will require further investigation.

Altogether, two canonical centrioles are present in many basal animal species with primitive form (aka aquatic sperm) (BACCETTI, 1985; Jamieson, 1991), indicating that atypical centrioles evolved independently at least eight times in animals

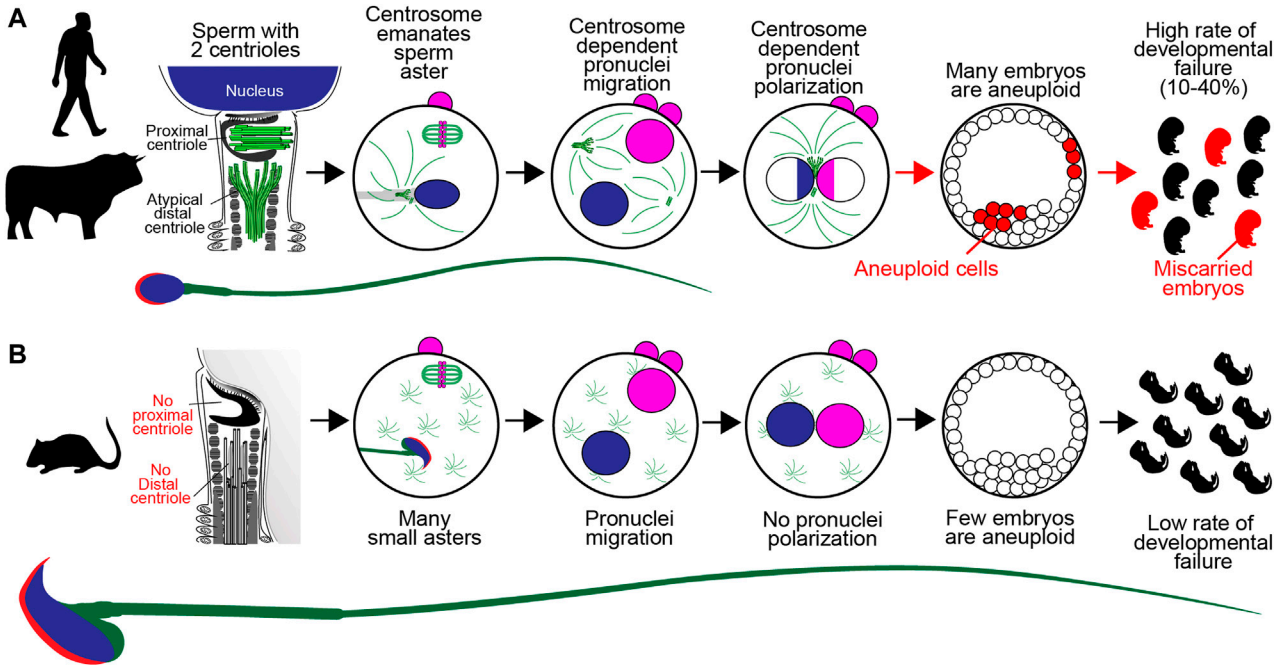

FIGURE 1 | A Model of Sperm Centriole Function Post-fertilization. **(A)** In human, bovine, and many other mammalian species, the sperm has two centrioles: the proximal centriole and atypical distal centriole. During fertilization, spermatozoan centrioles are brought into the oocyte, where they form the large sperm aster, or the zygotic centrosome, and which helps pronuclear migration. The initial, single zygotic centrosome breaks into two centrosomes that move to the interface between the two pronuclei, where they polarize the paternal genome (blue) and maternal genome (pink). Failure of the sperm centrioles (red arrow) or zygotic centrosome results in embryo aneuploidy (red x) and embryo developmental failure, which are common in human and bovine. **(B)** In mice, the sperm has no detectable centrioles. After fertilization, the many small, acentriolar, cytoplasmic asters in the oocyte continue to function. The pronuclear genomes are homogenous. Embryo aneuploidy is very low, and embryo development is robust.

and may represent convergent evolution. However, the selective force underlying this convergence is unclear. One common feature of animal groups with atypical sperm centrioles is internal fertilization. The sperm of animals with internal fertilization show greater divergence in structure and morphology (Baccetti, 1986; Kahrl et al., 2021), indicating that atypical centriolar diversity may have coevolved with diverging sperm structure and morphology. To test this hypothesis, it would be essential to determine the specific contribution of atypical centrioles to sperm physiology.

Sperm Centrioles Form a Dynamic Basal Complex in Bovine

As discussed above, a critical question is: why do sperm have an atypical centriole? The answer for that may vary among animal groups since each evolved independently. Revisiting our leg/centriole analogy, forelegs in basal mammals can become hands in humans or wings in bats (Krubitzer and Seelke, 2012); they were lost in the pectoral fins of dolphins (Cooper et al., 2007) and became flippers in sea lions (Reidenberg, 2007). Recently, we studied in detail the bovine atypical centriole and found that, together with other sperm neck structures, it forms a mechanical link that couples tail beating, and head kinking motions. This mechanical link, called the dynamic basal complex, (DBC) (Khanal et al., 2021) is comprised of the sperm's specialized PCM (the striated column and capitulum), the PC, and the atypical DC.

In mammals, the transfer of force from sperm tail to head involves several neck components that work together. The striated column and DC connect to the tail independently from each other, then interact with the PC and capitulum. Finally, the PC and capitulum form a complex attached to the head. Since the insect PC is atypical (proximal centriole-like (PCL) structure), one question that arises is whether it serves the same function as it does in mammals (Gottardo et al., 2015; Khire et al., 2016). This is not likely the case in *Drosophila*, as the DC connects directly to the tail axoneme and the head, while the PCL is found in the sperm neck cytoplasm to the side of the DC, and does not appear to be part of the same mechanical linkage. However, the location of the PCL just above the mitochondria derivative that spans the tail in parallel to the axoneme may allow the PCL to connect the tail to the head in parallel to the axoneme/DC/head linkage.

Another question is: why does the sperm DC show such large differences in size between species with centriolar reproduction even though the overall sizes of their sperm are similar? For example, the bovine DC is twice as long as the human DC, based on rod protein length (Khanal et al., 2021). This situation may be analogous to some mammals having short legs and others having long legs. While leg size tends to correlate with animal size, this is not always the case. The jerboa has longer legs than other rodents of similar size so that they can move more quickly and avoid predators (Chan, 2015). One possibility is that modifications to centriolar structure allow the DBC to adapt to the unique challenges of the female reproductive tract

(FRT). For example, unlike human and bovine, rabbit have induced ovulation, and the sperm reach the site of fertilization before the eggs (Fischer et al., 2012). Humans have a vagina connected to a central-chamber uterus (Suarez and Pacey, 2006), bovine have a vagina that is connected to a uterine body that splits into two uterine horns (Tung and Suarez, 2021), and rabbits have a vagina connected to two uteruses (a bicornuate duplex uterus) (Fischer et al., 2012). This diversity in reproductive anatomy may impose complex evolutionary pressures that modify sperm physiology and centriolar structure/function.

Sperm Centriolar Defect Contributes to Infertility and Miscarriages

Since centrioles are present in mature sperm and function in the zygote, they may have a critical role in fertility, and normal embryo development. The dogma in human reproductive biology is that sperm centriolar dysfunction will result in fertilization failure (Asch et al., 1995; Simerly et al., 1995; Rawe et al., 2002). This idea arises from the early role of sperm centrioles in forming a sperm aster that facilitates male and female pronuclear congregation (Rawe et al., 2002). Sperm with severely abnormal centriolar structure fail fertilization (Chemes et al., 1987; Chemes et al., 1999; Chemes and Alvarez Sedo, 2012). Still, sometimes, polyspermic oocytes can form bipolar spindles with multiple clustered centrosomes at their poles and divide normally (Wentz et al., 1983). This idea also extends from the fact that fertilization of the egg by multiple sperm results in centriole-induced multipolar spindles and abnormal cleavage (Kai et al., 2021). However, with mild centriolar defect (i.e., centrioles are present but are partially impaired), zygote division can continue, but aneuploidy can occur, resulting in embryo death at a later stage (Cavazza et al., 2021).

Considering that the zygotic centrosome is a composite of sperm centrioles, male centrosomal proteins, and egg PCM proteins, the male and female contributions may compensate for each other's deficiencies (Schatten, 1994; Amargant et al., 2021). Therefore, having mildly abnormal sperm centrioles can also be exacerbated by weakened eggs, resulting in failed embryonic development. This may explain why donor sperm particularly benefits older women in some cases (Bortoletto et al., 2021).

CONCLUSION

The precise role of centrioles in the sperm and early embryo remains enigmatic due to challenges in its study. However, recent data suggest that the sperm provides two functional centrioles needed for normal chromosome segregation during cleavages and maintenance of normal early embryo development in human and bovine. For that, the sperm centrioles recruit maternal egg proteins and form zygotic centrosomes, which interact with paternal chromosomes. This process is sensitive, and minute errors could cause alterations in centrosome function, aneuploidy, and miscarriages. More research is needed to confirm this recent observation and understand their mechanism and role in miscarriages in human, bovine, and additional mammalian models. Mouse embryos may have found a way to develop independently of centrioles, possibly by enhancing the alternative mechanisms that exist also in the human zygote, thereby eliminating the dependency on sensitive processes and simultaneously increasing the robustness of embryonic development and fecundity. However, little is known about how this independence was achieved. More data is needed on centriole status and miscarriage rates in other species, specifically in the rodent lineage that led to house mice. More research is required to develop methodologies to overcome research challenges and test this hypothesis.

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

TA-R wrote the first draft of the manuscript. LA and RU wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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Aberrant SKP1 Expression: Diverse Mechanisms Impacting Genome and Chromosome Stability

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The S-phase Kinase-Associated Protein 1 (SKP1) is a core component of the SKP1, Cullin 1, F-box protein (SCF) complex, an E3 ubiquitin ligase that serves to poly-ubiquitinate a vast array of protein targets as a signal for their proteasomal degradation, thereby playing a critical role in the regulation of downstream biological processes. Many of the proteins regulated by SKP1 and the SCF complex normally function within pathways that are essential for maintaining genome stability, including DNA damage repair, apoptotic signaling, and centrosome dynamics. Accordingly, aberrant SKP1 and SCF complex expression and function is expected to disrupt these essential pathways, which may have pathological implications in diseases like cancer. In this review, we summarize the central role SKP1 plays in regulating essential cellular processes; we describe functional models in which *SKP1* expression is altered and the corresponding impacts on genome stability; and we discuss the prevalence of *SKP1* somatic copy number alterations, mutations, and altered protein expression across different cancer types, to identify a potential link between SKP1 and SCF complex dysfunction to chromosome/genome instability and cancer pathogenesis. Ultimately, understanding the role of SKP1 in driving chromosome instability will expand upon our rudimentary understanding of the key events required for genome/chromosome stability that may aid in our understanding of cancer pathogenesis, which will be critical for future studies to establish whether SKP1 may be useful as prognostic indicator or as a therapeutic target.

Keywords: cancer, centrosome dynamics, chromosome instability, DNA damage response, Fbox protein, genome instability, SCF complex, SKP1

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INTRODUCTION

The SKP1 (S-phase Kinase-Associated Protein 1), CUL1 (Cullin 1), F-box protein complex (SCF complex) is an E3 ubiquitin ligase that regulates a vast array of cellular processes (e.g., cell cycle, DNA damage response, apoptosis and centrosome homeostasis) that are key to maintaining genome stability and ensuring proper segregation of genetic material into daughter cells. SKP1 is an invariable, core component of the SCF complex that functions as the adaptor protein responsible for binding CUL1 and recruiting various F-box proteins for SCF complex formation. This critical role of SKP1 enables the poly-ubiquitination of a diverse array of substrates targeted by the variable F-box proteins for subsequent proteolytic degradation by the 26S proteasome, making SKP1 activity essential to regulate the myriad of cellular processes governed by the SCF complex. Accordingly, genetic aberrations altering SKP1 expression and/or function will adversely impact the

many biological processes normally required to maintain genome stability, and thus aberrant *SKP1* expression is predicted to contribute to cancer pathogenesis. In support of this possibility, somatic alterations in *SKP1*, including mutations, deletions and mRNA misexpression occur frequently in a wide variety of cancer types.

Despite the many associations between altered *SKP1* expression and cancer, the fundamental impact aberrant SKP1 expression and/or function has on oncogenesis remains unclear. This review describes how aberrant SKP1 expression and function impacts many biological pathways that are essential to maintain genome instability that when altered, are implicated in oncogenesis. Accordingly, these observations support the possibility that aberrant *SKP1* expression may be a contributing pathogenic event, although definitive empirical data are still needed. First, we provide a historical background of mammalian SKP1, describing key characteristics at the gene/protein level as well as its relationship with orthologs from other species. We then discuss how SKP1 interacts with the other SCF complex members and their collective role within the ubiquitin proteasome system (UPS). Next, we describe the roles that SKP1 and the SCF complex have within three biological processes that are essential for maintaining genome stability, an enabling hallmark of cancer (Hanahan and Weinberg, 2011) including: 1) altered DNA damage response and apoptosis; 2) aberrant centrosome duplication and dynamics; and 3) chromosome stability. To further support a potential role in cancer pathogenesis, we detail the occurrence and frequency of *SKP1* alterations within cancer patient samples. Finally, we conclude with a brief discussion on future therapeutic strategies that seek to exploit altered *SKP1* expression and the downstream impacts of aberrant protein targeting and destruction.

SKP1—A HISTORICAL PERSPECTIVE AND FUNDAMENTAL PROPERTIES

Mammalian SKP1, also referred to as the Cyclin-A/Cyclin Dependent Kinase (CDK) 2-Associated Protein 19 (P19), was originally identified in 1980 within the guinea pig organ of corti by 2D polyacrylamide gel electrophoresis and was consequently named Organ of Corti Protein 2 (OCP2) (Thalmann et al., 1980; Thalmann et al., 2003). In the 1990s, a series of research groups independently investigated *SKP1/P19* and its aliases *OCP2* and *TCEB1L* as seemingly distinct genes. In 1995, Zhang and others (Zhang et al., 1995) determined that human SKP1/P19 interacted with the Cyclin A/CDK2 complex, suggesting a potential role in cell cycle regulation, and subsequently sequenced the *SKP1/P19* DNA coding regions. Concurrently, Chen *et al* (Chen et al., 1995) sequenced human *OCP2*, while Sowden *et al* (Sowden et al., 1995) presented the cDNA sequence for a novel gene designated *TCEB1L*, suspected to encode a transcription elongation factor. Additionally, Bai and others (Bai et al., 1996) identified the yeast and human orthologs of SKP1 as a suppressor of cdc4 (cell division control 4) and as a Cyclin F-binding protein, respectively, in two independent lines of research. It was not until 1997, when Liang *et al* (Liang et al., 1997) noted that the coding sequences detailed above for human *SKP1/P19*, *OCP2*, and *TCEB1L* were identical and that the above genes encoding distinct roles in diverse cellular processes were in fact, one and the same.

The human *SKP1* gene spans a region of 28,097 base pairs (bp) on chromosome 5q31.1 and encodes two protein coding mRNA transcripts of different lengths, 2,028 bp and 2,714 bp that are generated by alternative splicing. The transcripts are translated into two protein isoforms, 163 (Isoform B) and 160 (Isoform A) amino acids in size that differ at their carboxy-terminal regions (**Figure 1A**) (2009). Although Isoform B is considered the prototypic SKP1 protein (Schulman et al., 2000; Yamanaka et al., 2002; Kong et al., 2004), the potential functional differences between the two isoforms have yet to be fully explored. Nevertheless, a study in *Saccharomyces cerevisiae* revealed that the tryptophan residue at position 159 (Trp159), present only in human Isoform B (**Figure 1A**), is essential for its *in vivo* function. As Trp159 is evolutionarily conserved from yeast to humans, these experimental findings in *S. cerevisiae* suggest there may only be one functional human isoform (i.e., Isoform B) (Schulman et al., 2000). To test this possibility, isoform-specific studies must be designed to formally interrogate the functional differences and discern whether the non-prototypic SKP1 Isoform A has developed a *de novo*, Trp159-independent function during evolution.

SKP1 Isoform B (**Figure 1B**) is ~18 kDa and harbors a 128 residue domain at the amino-terminus resembling the α-helix/β-sheet structure of a BTB/POZ (broad complex, tramtrack and bric-à-brac/poxviruses and zinc finger) fold domain, but with an α-helical insertion (αH4) (Schulman et al., 2000). This domain is essential for heterodimerization and is required for the binding of SKP1 to the SCF complex scaffolding protein, CUL1. Additionally, SKP1 harbors a two-helix, carboxy-terminal extension (αH7 and αH48) that cooperates with elements of the BTB/POZ fold to create a variable interaction motif that binds F-box domains (**Figure 1B**). There are 69 distinct proteins containing F-box domains (i.e., F-box proteins) that have been identified in mammals (Jin et al., 2004), each with its own set of protein targets. Thus, SKP1 serves as an adaptor between CUL1 and one of 69 F-box proteins, playing a critical role in the formation of up to 69 distinct SCF complexes (**Figure 1C**) (Ng et al., 1998; Yoshida et al., 2011) and the regulation of a diverse set of protein targets and pathways.

EVOLUTION OF SKP1 SEQUENCE AND FUNCTION FROM MODEL ORGANISMS TO HUMANS

The amino acid sequences and structural elements of human SKP1 share a significant degree of amino acid sequence similarity with its counterparts in model organisms including *S. cerevisiae* (98% similar; 43% identical), *Mus musculus* (100% similar; 99% identical), *Drosophila melanogaster* (100% similar; 77% identical), *Caenorhabditis elegans* (97% similar; 71% identical) and *Arabidopsis thaliana* (71% similar; 58% identical) (2009). Beyond these sequence and structural similarities, functional conservation is also readily apparent between humans and

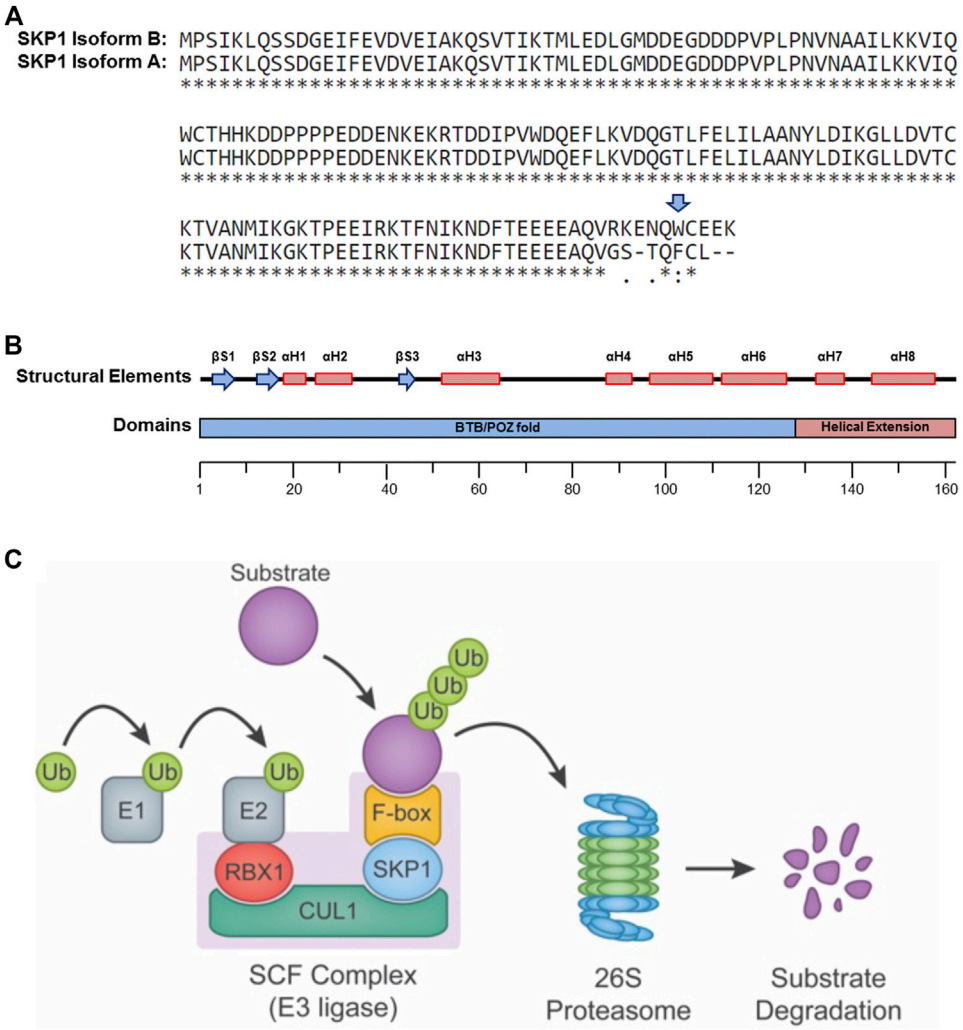

FIGURE 1 | SKP1 Structure and Function. **(A)** Single amino acid sequence alignment of the two SKP1 (isoform A and isoform B) reveals sequence divergence within their carboxy-terminal tails. Sequence alignments performed using UniProt (Universal Protein Resource) (Altschul et al., 1990). Tryptophan 159 (W159), present only within Isoform B is highlighted by a blue arrow. A "*" identifies conserved amino acid positions, while ":" and "." identify amino acid positions with similar or weakly similar properties, respectively. **(B)** Schematic depiction for the secondary structural elements (top) and protein domains (bottom) of SKP1 isoform B (βS, beta-sheet; αH, alpha-helix; BTB/POZ, broad complex, tramtrack and bric-à-brac(BTB)/poxviruses and zinc finger (POZ)). **(C)** Diagram depicting the SCF complex and its function in targeting protein substrates for poly-ubiquitination and proteolytic degradation by the 26S proteasome. The SCF complex consists of three invariable components (RBX1, CUL1, and SKP1) and one of 69 variable F-box proteins that confers substrate specificity. In general, ubiquitin (Ub) moieties are transferred to a protein substrate through the sequential actions of an E1 (activating) and an E2 (conjugating) enzymes in conjunction with an E3 (ligase) enzyme (e.g., SCF complex).

model organisms. For example, human *SKP1* has been shown to functionally compensate for *Skp1* deletion in *S. cerevisiae* (Bai et al., 1996). Although only one functional isoform is proposed to exist in humans, studies in *C. elegans* have identified at least 21 *SKP1* paralogs or *Skp1*-related genes, each exhibiting varying degrees of sequence similarity with human *SKP1* (Yamanaka et al., 2002). Similarly, *D. melanogaster* and *A. thaliana* harbor 7 and 19 *Skp1*-related genes, respectively (Yamanaka et al., 2002; Kong et al., 2004), which exhibit tissue-specific expression and unique binding specificities for both F-box and Cullin-family proteins. Furthermore, while the role of the shorter human SKP1 Isoform A has not yet been well-characterized, it remains possible that Isoform A may recognize alternate F-box proteins or be involved in SCF complex-independent functions. In general, the high degree of sequence and functional conservation throughout evolution underscores the key role SKP1 plays within the SCF complex and further emphasizes the importance of SKP1 in the regulation of fundamental cellular processes.

SKP1 IS A CORE COMPONENT OF THE SCF UBIQUITIN LIGASE COMPLEX AND THE UBIQUITIN PROTEOSOME SYSTEM

SKP1 and the SCF complex are arguably best understood for their roles in poly-ubiquitination, proteolytic degradation and the

UPS. The UPS is a highly coordinated series of events involving the covalent attachment of ubiquitin molecules to protein targets and the subsequent degradation of these poly-ubiquitinated targets by the 26S proteasome. Substrate poly-ubiquitination is accomplished through the successive and repeated activities of three key enzymes (**Figure 1C**) that are generically referred to as the E1 ubiquitin (activating) enzyme, the E2 ubiquitin (conjugating) enzyme and the E3 ubiquitin (ligating) enzyme (reviewed in (Hershko and Ciechanover, 1998; Nakayama and Nakayama, 2006; Deshaies and Joazeiro, 2009)). Approximately 600–650 E3 ligases are predicted to exist within humans, which impart the extensive and requisite specificities to regulate the hundreds to thousands of protein targets believed to be modulated by the UPS, whereas only two E1 and approximately thirty E2 enzymes exist within the human genome (Deshaies and Joazeiro, 2009).

The E3 ubiquitin ligases are classically divided into three main families based on distinct structural motifs and include: 1) the Really Interesting New Gene (RING)-finger family containing ~600 members in humans; 2) the Homologous to the E6-AP Carboxyl Terminus (HECT) family having ~30 human members; and 3) the RING-between RING-RING (RBR) family with ~12 members in humans (Morreale and Walden, 2016). The RING-finger family is further divided into sub-families, which includes the Cullin-RING ligase subfamily. The SCF complex is often considered the prototypic Cullin-based RING-finger E3 ubiquitin ligase and is comprised of three invariable core components (**Figure 1C**): 1) the RING-finger protein RBX1 (Ring-Box 1, also known as the regulator of cullins 1 [ROC1]) that recruits the E2 ubiquitin-conjugating enzyme; 2) CUL1, a scaffolding protein that complexes the E2 to the SCF complex; and 3) SKP1, the adaptor protein that physically connects the F-box protein and corresponding protein target with the core SCF complex.

F-box proteins are classified into three distinct families according to their substrate recognition domains, namely FBXW, FBXL, and FBXO family members, which harbor WD40 repeats (e.g., FBXW7), leucine-rich repeats (e.g., FBXL1/SKP2) or other domains (e.g., FBXO28), respectively, (Jin et al., 2004). As indicated above, it is the F-box protein that imparts the protein target specificity to the SCF complex, with F-box proteins often binding to phospho-activated targets. Once bound to the protein target, the F-box protein/protein target are subsequently recruited to the core SCF complex through an interaction with SKP1 to enable the transfer of ubiquitin from an E2 conjugating enzyme onto the protein target. It is the repeated covalent attachment of ubiquitin moieties (i.e., poly-ubiquitination) via specific linkages (lysine 48 [K48] linkages) that label the designated substrates for degradation by the 26S proteasome. Thus, it is the UPS that regulates the global and temporal abundance of an extensive array of protein targets within a given cell (Kulathu and Komander, 2012).

While there are potentially 69 distinct SCF complexes, the substrates and functions for many of these SCF complexes remain largely unknown. Nevertheless, there are a few well characterized F-box proteins/SCF complexes that target key proteins involved in a variety of cellular pathways such as DNA damage repair, apoptosis, centrosome biology and chromosome stability (discussed below), which highlights their innate roles in maintaining genome stability and preserving mitotic fidelity. As such, future studies aimed at functionally characterizing the complete cellular repertoire of SCF complexes will be essential to advance our rudimentary understanding of the specific impact each individual SCF complex has in normal cell physiology and genome stability. Perhaps even more important will be the fundamental and clinical studies aimed at determining the impact aberrant expression and function of SCF complex components have on disease development. Indeed, aberrant SKP1 expression and/or function is already associated with several human genetic disorders, including Sjögren's syndrome (a chronic inflammatory autoimmune disease) (Sandhya and Danda, 2014), sporadic Parkinson's disease (a neurological degenerative disorder) (Mandel et al., 2012) and cancer (Silverman et al., 2012). Thus, defining the underlying molecular etiology giving rise to SKP1 (and SCF complex) dysfunction will be critical to ultimately determine the individual and collective impacts on disease pathology, especially as it potentially relates to cancer development and progression.

SKP1 AND THE SCF COMPLEX COORDINATE THE DNA DAMAGE RESPONSE AND APOPTOSIS

The processes that regulate cell cycle progression and DNA damage response are intimately linked and are essential to maintain genome stability. In the presence of genotoxic stress or a stalled replication fork, a cell cycle arrest is invoked to facilitate repair prior to cell cycle re-entry with the ultimate goal of preventing genomic damage (mutations and alternations) from being propagated within daughter cells (Bassermann et al., 2014). These processes are highly dependent on appropriate protein turnover that is regulated by the UPS. Indeed, the SCF complex, and therefore SKP1, exhibit key roles within the DNA damage response, some of which are detailed below.

In general, following a DNA double strand break, a checkpoint kinase, either ATM (Ataxia Telangiectasia Mutated) or ATR (Ataxia Telangiectasia and Rad3 Related) is auto-phosphorylated, which initiates a series of cascading phosphorylation events on downstream targets. For example, ATM initiates a G1 arrest by phosphorylating Cyclin D1, which is subsequently ubiquitinated by SCF^{FBXO4} and targeted for proteolytic degradation. In turn, Cyclin D1 degradation promotes CDK2 inhibition by releasing P21 from CDK4 (Agami and Bernards, 2000), which ultimately prevents E2F transcription factor activation and cyclin expression (Silverman et al., 2012). Alternatively, an S-phase or G2 arrest can be invoked through ATR phospho-activation of CHEK1 (Checkpoint Kinase 1), which is mediated by the adaptor protein Claspin (Mamely et al., 2006) to hyperphosphorylate CDC25A, labeling it for $SCF^{\beta TrCP(FBXW11)}$ mediated targeting and proteolytic degradation to attenuate CDK activation (Busino et al., 2003). This CDK attenuation induces a cell cycle arrest,

providing the requisite time for efficient DNA repair. Moreover, to ensure an adequate supply of deoxyribonucleotides for DNA repair, degradation of RRM2 (Ribonucleotide Reductase Regulatory Subunit 2) via $SCF^{CyclinF(FBXO1)}$ is inhibited by ATR-mediated Cyclin F degradation (D'Angiolella et al., 2012). Concurrently, the pre-replication complex component, CDT1 (Chromatin Licensing and DNA Replication Factor 1) is targeted for degradation by $SCF^{SKP2(FBXL1)}$ to prevent replication of damaged DNA (Kondo et al., 2004), while protein translation is reduced by the phospho-inactivation of the elongation factor, eEF2 (Eukaryotic Translation Elongation Factor 2) by eEF2K to prevent unnecessary energy expenditure during the DNA damage response. Once DNA repair is complete, $SCF^{\beta TrCP}$ directs eEF2K degradation to rapidly resume protein synthesis (Kruiswijk et al., 2012). $SCF^{\beta TrCP}$ also coordinates cell cycle re-entry by targeting phosphorylated Claspin for degradation, preventing CHEK1 activation by ATR, allowing for CDC25A reactivation of CDKs, while the increased abundance of CHEK1 is reduced by targeted degradation mediated by SCF^{FBXO6} (Silverman et al., 2012; Bassermann et al., 2014).

As the SCF complexes described above are crucial for DNA damage repair and maintaining genome stability, it is not difficult to envision how mutation, aberrant expression and/or function of SKP1 promotes genome instability and may contribute to cancer development and progression. For example, the siRNA-based silencing of βTrCP in S-phase cells exposed to ionizing radiation results in CDC25A accumulation (Jin et al., 2003), a defective S-phase check-point, failure to inhibit DNA replication and the propagation of DNA damage underlying genome instability and cancer (Bassermann et al., 2014).

In the event of excessive DNA damage, apoptosis is typically initiated to remove those cells from the population and prevent transmission of damaged DNA to daughter cells, which is a process normally regulated by the SCF^{FBXW7} complex. In response to DNA damage, GSK3 (Glycogen Synthase Kinase 3) phosphorylates the anti-apoptotic BCL2 (B-Cell Chronic Lymphocytic Leukemia/Lymphoma 2) family member MCL1 (Myeloid Cell Leukemia 1), allowing for SCF^{FBXW7}-mediated MCL1 poly-ubiquitination and degradation. The cell death promoters BAX (BCL2 Associated X Protein) and BAK (BCL2 Antagonist/Killer) are released from MCL1 inhibition, which stimulates mitochondrial membrane permeabilization, caspase activation and apoptosis induction. Deletion of *FBXW7* or its functional inactivation in acute lymphoblastic leukemia (ALL) cells, impairs MCL1 degradation in response to DNA-damaging agents, resulting in MCL1 overexpression and evasion of apoptosis (Inuzuka et al., 2011). In support of a role in oncogenesis, *FBXW7* is somatically altered in >30% of human T-cell lymphomas, while T-cell-specific *Fbxw7* knockout mice develop ALL (Crusio et al., 2010). Moreover, ~20% of patients with colorectal adenocarcinoma have somatic *FBXW7* mutations (Tate et al., 2019), with altered *FBXW7* expression contributing to tumor development and progression, while loss-of-function mutations are predicted to be deleterious. Furthermore, ~50% of somatic *FBXW7* mutations occur at three hotspot codons (Arg465; Arg479; Arg505), which disrupt binding of FBXW7 to target substrates (Akhoondi et al., 2007; Cancer Genome Atlas Network, 2012; Grim, 2014), highlighting the critical role of the SCF complex and how dysregulation of key components may contribute to oncogenesis. Collectively, the above data demonstrate that SKP1 and the SCF complex are critical for coordinating a cellular response to DNA damage and facilitating either DNA repair or apoptosis depending on the extent of the damage.

As SKP1 is an invariable component of each SCF complex described above, *SKP1* alterations such as mutations or copy number alterations (gains or losses) are predicted to impede DNA damage repair and foster cell survival by adversely impacting pro-apoptotic pathways leading to genome instability and perhaps promoting oncogenesis. This possibility is supported by the work of Piva and others (Piva et al., 2002), who generated and employed a transgenic mouse expressing a *Cul1* deletion mutant (Cul1-N252) that sequesters and inactivates murine Skp1 (discussed further below). Interestingly, the *in vivo* inhibition of Skp1 function in a T-cell lineage corresponded with the development of T-cell lymphomas. Upon closer scrutiny, the authors also noted significant increases in micronucleus formation (DNA containing, extranuclear bodies indicative of DNA damage and genome instability (Bhatia and Kumar, 2013)), centrosome abnormalities, aberrant chromosome segregation and karyotypic heterogeneity. These data suggest SKP1 is critical to preserve the function of essential biological processes (e.g., DNA repair and apoptosis), while aberrant SKP1 expression and/or function disrupts these essential processes in a manner that may promote oncogenesis. Thus, it will be of tremendous interest to determine whether the accumulation of DNA damage within *SKP1*-deficient cancer cells or appropriate mouse models are associated with increased sensitivity towards genotoxic agents or whether these cells/models can be selectively targeted with immune checkpoint inhibitors or precision-based therapeutic strategies.

SKP1 AND THE SCF COMPLEX REGULATE CENTROSOME DYNAMICS

To ensure the accurate and faithful transmission of genetic material to daughter cells, chromosome dynamics are tightly regulated by the UPS, which coordinates centriole/centrosome duplication and separation. Centrosome aberrations lead to ongoing chromosome missegregation events and aneuploidy that are frequently observed in a myriad of cancer types. For example, one immunohistochemical study (Pihan et al., 1998) revealed that 93% (81/87 total) of human breast, prostate, lung, colon, brain, and metastatic cancer samples exhibit abnormal centrosome phenotypes including aberrant size, shape, and numbers relative to those in noncancerous adjacent tissues. Moreover, the aberrant phenotypes observed in tumor-derived cell lines are correlated with CIN (chromosome instability), a common form of genome instability characterized by ongoing changes in chromosome number and/or structure that is an established driver of cell-to-cell and genetic heterogeneity (reviewed in (Geigl et al., 2008; Lepage et al., 2019; Vishwakarma and McManus, 2020)). More recent studies have

determined that SKP1 localizes to the centrosome throughout the cell cycle and that $SCF^{CyclinF}$ (D'Angiolella et al., 2010), SCF^{FBXW5} (Puklowski et al., 2011) and $SCF^{\beta TRCP}$ (Chan et al., 2008) exhibit key roles in centrosome dynamics (Gstaiger et al., 1999; D'Angiolella et al., 2010) that when disrupted with proteasome inhibitors (MG132), adversely impact centrosome formation and duplication. For example, during G2, the centriolar protein CCP110 (Centriolar Coiled-Coil Protein 110) that normally promotes centriole replication while inhibiting elongation, is targeted for proteolytic degradation by $SCF^{CyclinF}$ (Chen et al., 2002). Such timely CCP110 degradation prevents centriole over-duplication that would otherwise result in supernumerary centrosomes, chromosome missegregation events and aneuploidy. Indeed, D'Angiolella and others (D'Angiolella et al., 2010) determined that Cyclin F silencing induces centrosome over-duplication in G2 leading to multi-polar spindle formation, lagging chromosomes and an increase in micronucleus formation, all of which are hallmarks of CIN (Geigl et al., 2008; Lepage et al., 2019; Vishwakarma and McManus, 2020). As expected, co-silencing Cyclin F and CCP110 rescues these aberrant phenotypes effectively confirming the underlying mechanism leading to their formation.

Beyond CCP110, the centriolar scaffolding protein SASS6 (Spindle Assembly Protein 6) is also essential for centrosome formation and duplication, and is degraded in G2 by SCF^{FBXW5}, which prevents over-duplication of centrosomes. FBXW5 is negatively regulated by APC/C (Anaphase-Promoting Complex/Cyclosome) and PLK4 (Polo-Like Kinase 4), which enables SASS6 to function appropriately during G1 and S-phase, respectively. As predicted, reduced *FBXW5* expression corresponds with increasing SASS6 abundance and abnormally increased numbers of centrioles (Puklowski et al., 2011). Similarly, PLK4 promotes centriole duplication and separation, and is tightly regulated by $SCF^{\beta TRCP}$ (Guderian et al., 2010). Thus, aberrant PLK4 expression is associated with aberrant centriole numbers in human cancer cells (Habedanck et al., 2005), while *βTrcp1* knockout in mouse embryonic fibroblasts corresponds with centrosome over-duplication and supernumerary centrosomes (Guardavaccaro et al., 2003). $SCF^{\beta TRCP}$ also contributes to centrosome homeostasis and chromosome stability by regulating the degradation of BORA (BORA Aurora Kinase A Activator), an activator Aurora Kinase A (AURKA). BORA regulates AURKA localization and kinase activity at the centrosome to ensure proper centrosome and mitotic spindle development, as overexpression of a $SCF^{\beta TRCP}$-resistant form of BORA interferes with bipolar spindle formation as it adversely impacts AURKA localization and function (Chan et al., 2008). Based on these few examples, it is apparent that SKP1 and the SCF complex are critical for regulating centrosome dynamics and function, which is essential for chromosome transmission fidelity. Thus, further clinical studies into the types and prevalence of genomic aberrations affecting SKP1 expression are essential to better understand their impact on centrosome biology and gain a more holistic understanding of the potential downstream implications for disease development.

ABERRANT *SKP1* EXPRESSION INDUCES CIN THAT MAY PROMOTE ONCOGENESIS

As an invariable component of the SCF complex, it is apparent that SKP1 is essential for the proper regulation of key substrates involved in many cancer-associated pathways. Despite this association, the potential pathophysiological impact aberrant *SKP1* expression may have in cancer development is only beginning to emerge. This knowledge gap may in part, be attributed to the lack of transgenic or *Skp1* knockout mouse models available for *in vivo* study (Zhou et al., 2013). Nevertheless, several transgenic mouse models do exist for the other SCF complex components (e.g., Cul1) that have provided key insight into SKP1 (and SCF complex) function, which includes the pathogenic implications for genomic instability and cancer associated with aberrant SCF complex expression and function. As indicated above, Piva *et al.* (Piva et al., 2002) developed a *Cul1* deletion mutant (Cul1-N252) transgenic mouse model that inactivates Skp1 *in vivo*, leading to lymphoid organ hypoplasia, proliferation defects, supernumerary centrosomes, mitotic spindle aberrations and CIN. Following the initial proliferation reduction, >80% of Cul1-N252 mice develop T-cell lymphomas, suggesting Skp1 and SCF function are required to prevent lymphoid tumor development. Moreover, Cul1-N252 expression in a human cellular context (HEK293T cells) resulted in many aberrant phenotypes associated with CIN, including multinucleated cells, enlarged nuclei and increased micronucleus formation. Thus, their mouse and human work are consistent with aberrant Skp1/SKP1 function being an early etiological event underlying CIN and possibly contributing cancer pathogenesis. Moreover, these results highlight the utility of mouse models for studying the *in vivo* functions of SCF components and provide a means by which to investigate their potential roles in tumorigenesis. Their findings also underscore the paucity of clinically-relevant *Skp1* mouse models, which are essential to clearly delineate and characterize any potential role for aberrant *Skp1*/*SKP1* expression and/or function in oncogenesis.

Recently, several genetic studies have begun to identify potential pathogenic relationships between aberrant SCF complex expression/function and cancer (Thompson et al., 2020; Bungsy et al., 2021; Lepage et al., 2021). In particular, two studies focused on the impact reduced *SKP1* expression has on CIN in colorectal (Thompson et al., 2020) and ovarian (Lepage et al., 2021) cancer contexts. First, Thompson *et al* (Thompson et al., 2020) performed a screen of 164 candidate genes whose diminished expression was suspected to underlie CIN. Using siRNA-based silencing and quantitative imaging microscopy, they determined that reduced *SKP1* expression induced significant increases in CIN-associated phenotypes (Lepage et al., 2019), such as nuclear areas, micronucleus formation and chromosome numbers. They further showed that *SKP1* silencing corresponded with increases in replication stress, DNA double strand breaks and chromothriptic events, or extensive chromosome shattering followed by reassembly in a single event (reviewed in (Ly and Cleveland, 2017)). Perhaps most importantly, they performed genetic rescue experiments and

determined that the aberrant phenotypes were largely dependent on aberrant increases in Cyclin E1 levels, an established target of the SCF complex; however, as complete phenotypic rescues did not occur, they posited that additional protein targets must also be misregulated that contribute to the plethora of aberrant phenotypes observed. Given that ~85% of sporadic colorectal cancers exhibit CIN (Lengauer et al., 1997; Cisyk et al., 2015; Cisyk et al., 2018), these findings are particularly important as they may shed new insight into the potential underlying molecular etiology driving colorectal cancer pathogenesis. A second study by Lepage and others (Lepage et al., 2021), assessed the impact that reduced *SKP1* (and *CUL1*) expression has on CIN in non-transformed fallopian tube secretory epithelial cells, a cell of origin for high-grade serous ovarian cancer (Perets et al., 2013; Nakamura et al., 2018). Using a combination of siRNA and CRISPR/Cas9 approaches, they demonstrated that reduced expression corresponded with significant changes in nuclear areas, micronucleus formation and chromosome numbers. They further showed that CIN was prevalent and dynamic over an ~3-month timeframe, which is key given recent evidence showing that CIN is both pervasive and dynamic in ascites (an accumulation of abdominal fluid containing tumor cells) and solid tumor samples isolated from patients with high-grade serous ovarian cancer (Penner-Goeke et al., 2017; Morden et al., 2021). Collectively, these data identify *SKP1* as a novel CIN gene and further suggest that reduced expression may contribute to cancer pathogenesis. Accordingly, future fundamental and clinical studies are now essential to determine the extent and types of *SKP1* genetic alterations that may drive disease development and progression, with potential downstream implications for treatment response and patient outcomes.

SKP1 EXPRESSION IS FREQUENTLY ALTERED IN HUMAN CANCERS

As SKP1 and the SCF complex normally function to regulate a multitude of essential cellular pathways required to maintain genome stability, genetic alterations impacting the invariable complex components (e.g., *SKP1*) are anticipated to promote cellular dysfunction, which may contribute to cancer development. As detailed above, several genetic studies performed in both malignant (Thompson et al., 2020) and non-malignant (Lepage et al., 2021) human cell contexts have established that reduced *SKP1* expression induces CIN, an enabling hallmark of cancer (Hanahan and Weinberg, 2011) associated with cellular transformation, intra-tumoral heterogeneity, metastasis, drug resistance and poor patient outcomes (reviewed in (Geigl et al., 2008; Vishwakarma and McManus, 2020)). Unfortunately, *Skp1* knockout mice do not exist, suggesting it may be an essential gene, a possibility supported by a CRISPR screen that identified *SKP1* as an essential gene (Blomen et al., 2015); however, it should be noted that this work was conducted in a haploid malignant cancer cell line, and thus, the results may exhibit context-specific essentiality. Nevertheless, additional evidence comes from DepMap (Dependency Mapping), which is an online resource that identified *SKP1* a common essential gene based on RNAi and CRISPR screens performed in a myriad of cell lines (Tsherniak et al., 2017; Dempster et al., 2019; Dharia et al., 2021; Pacini et al., 2021). Accordingly, while *SKP1* appears to be an essential gene the functional impacts altered *SKP1* expression has on various biological pathways are only beginning to emerge (Thompson et al., 2020; Lepage et al., 2021).

In support of reduced *SKP1* expression and/or function harboring a potential pathogenic role in oncogenesis, *in silico* analyses of The Cancer Genome Atlas (TCGA) pan-cancer atlas patient data available through cBioPortal (Cerami et al., 2012; Gao et al., 2013) reveal that *SKP1* is somatically altered in 12 common solid tumor cancer types (**Figure 2**) (Hoadley et al., 2018). Briefly, *SKP1* mutations are rare with only 15 missense and 2 truncating mutations (one frameshift and one premature stop codon) identified within six of the 12 cancers assessed (**Figure 2A**) (Hoadley et al., 2018). Interestingly, and in agreement with *SKP1* being a putative tumor suppressor gene, the mutational load is equally distributed (i.e., diffuse) across the entire coding sequence (**Figure 2B**), rather than a focal mutational load that is typical of an oncogene (Liu et al., 2011; Vogelstein et al., 2013; Sato et al., 2015). With respect to gene copy number alterations, both gains (oncogene-like) and losses (tumor suppressor-like) occur in all 12 cancer types; however, losses are more prevalent in 11 of 12 cancers evaluated (**Figure 2C**). Overall, *SKP1* amplifications (two or more additional copies) are rare (0–1.0%), while gains (one additional copy) occur in all 12 cancers analyzed and range from 3.2 to 30.7% in uterine and liver cancers, respectively. Similarly, deep (i.e., homozygous) deletions are rare (0–1%), whereas shallow (i.e., heterozygous) deletions are present in all 12 cancer types and range from 6.3 to 43.8% in prostate and ovarian cancers, respectively. Collectively, these data show that large copy number alterations (amplifications or deep deletions) are rare, which suggests an expression threshold may exist whereby too much expression (i.e., gene amplification) may severely impact normal cellular physiology. Furthermore, complete loss (i.e., deep deletion) appears incompatible with viability further supporting the notion that *SKP1* is an essential gene (Blomen et al., 2015; Tsherniak et al., 2017; Dempster et al., 2019; Dharia et al., 2021; Pacini et al., 2021).

A fundamental assumption of gene copy number alterations is that they induce corresponding changes in gene expression and that *SKP1* copy number gains and losses are expected to underlie aberrant SCF complex activity leading to cellular dysfunction, genome instability and potentially tumorigenesis. Indeed, strong positive correlations exist between copy number changes and mRNA expression for all 12 cancer types investigated (**Figure 3**), and while the copy number alterations detailed above suggest *SKP1* may encode both oncogene-like or tumor suppressor-like functions, these seemingly opposing activities are not specific to *SKP1* and have been reported for other genes including *TP53* (Lane, 1984; Jenkins et al., 1985; Finlay et al., 1989), *USP22* (Jeusset and McManus, 2017), and *RAD54B* (McAndrew and McManus, 2017).

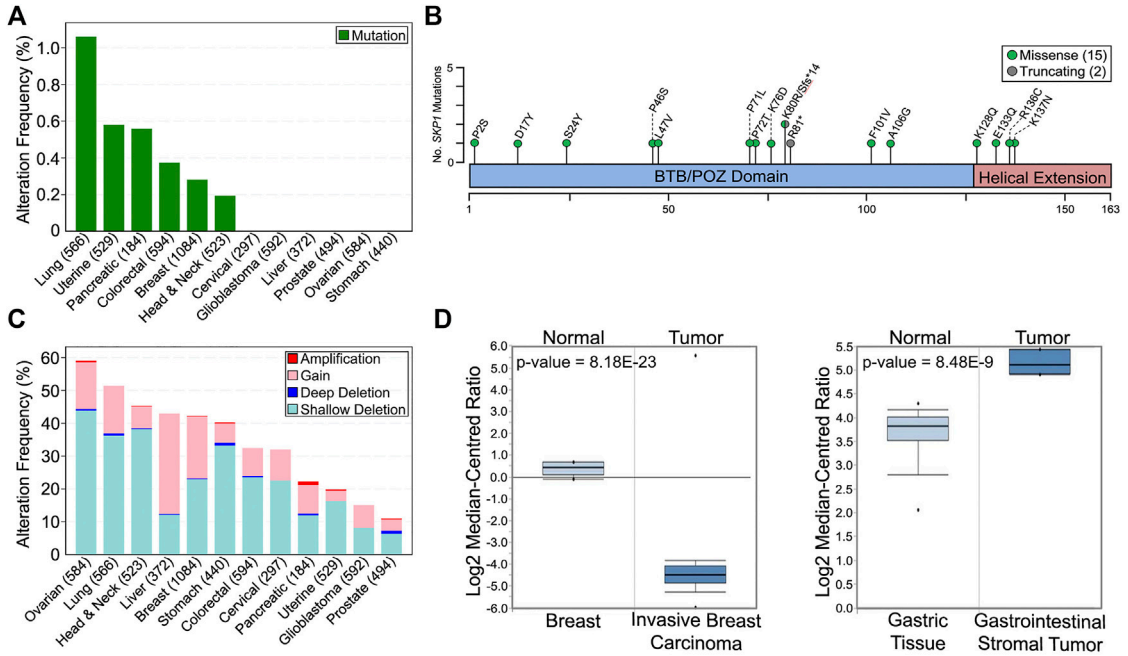

FIGURE 2 | Prevalence and Impact of *SKP1* Alterations in Cancer. **(A)** The frequency of total *SKP1* mutations (missense; truncating; inframe; fusion) in 12 common, solid tumor cancer types (total cases) (Cerami et al., 2012; Gao et al., 2013). Note that only missense (15) and truncating (2) mutations were identified from the 12 pan-cancer TCGA datasets (Hoadley et al., 2018). **(B)** Schematic mapping the positions of the encoded *SKP1* mutations across the SKP1 protein using the corresponding single amino acid codes (fs, frameshift; *, premature stop codon). **(C)** Prevalence of *SKP1* copy number alterations (deep deletion; shallow deletion; gain; amplification) within the 12 common cancer types (total cases) (Cerami et al., 2012; Gao et al., 2013; Hoadley et al., 2018). **(D)** Box-and-whisker plots displaying *SKP1* mRNA expression levels for normal and tumor tissues from invasive breast carcinoma (left) and gastrointestinal stromal tumor (right). Boxes display interquartile range, whiskers denote 10th and 90th percentiles, and the minimum/maximum values are displayed as black dots. Note that a significant >25-fold decrease in mean *SKP1* expression occurs in invasive breast carcinoma relative to normal tissue, while a significant ~3-fold increase in expression occurs in gastrointestinal stromal tumors. Data, graphs and statistical analyses were obtained from the Oncomine database (https://www.oncomine.org) (Rhodes et al., 2007).

The potential for *SKP1* to encode both tumor suppressor-like and oncogene-like activities is further bolstered by the many additional gene expression datasets available through various online resources. For example, while data contained within the *In Silico* Transcriptomics Online database (https://ist.medisapiens.com) (Kilpinen et al., 2008) show tremendous variation in *SKP1* (ENSG00000113558) mRNA expression in both normal and tumor tissues (see (Thompson et al., 2021)), they also reveal that some cancers exhibit increases (head and neck; chronic lymphocytic leukemia; liver) or decreases (breast; ovarian; cervical; colorectal) in *SKP1* expression relative to the corresponding normal tissues. Additionally, expression data from the Oncomine database (https://www.oncomine.org) (Rhodes et al., 2007) corroborate that *SKP1* can be under or overexpressed within specific cancer types relative to normal tissues. For example, **Figure 2D** provides representative examples in which *SKP1* is predicted to encode both tumor suppressor-like functions, as mRNA expression is significantly reduced (~25-fold) within invasive breast carcinomas relative to normal tissues, or oncogene-like functions, as expression is significantly increased (~3-fold) within gastrointestinal stromal tumors. Collectively, the data presented above support the possibility that *SKP1* may encode either oncogene- or tumor suppressor gene-like capabilities depending on whether it is over or under-expressed, respectively.

Unfortunately, very little insight into *SKP1* expression is available beyond transcriptomics, as only a single study has been performed in which SKP1 was assessed at the protein level. In 2015, Liu and others (Liu et al., 2015) employed western blots (64 matched cases) and immunohistochemistry (20 matched cases) to investigate SKP1 expression in non-small cell lung cancer and adjacent normal lung tissues. While both approaches revealed variable SKP1 expression in both cancer and matched tissues, 56% of cases showed significant increases in expression within tumors relative to control tissues. Furthermore, they determined that SKP1 expression was inversely correlated with survival as patients with high expression levels had significantly worse overall survival than those with low expression levels; however, the thresholds defining high versus low were not specified. Although the underlying genomic defects accounting for the increases in SKP1 expression observed in this study were not determined, this single example supports the possibility that aberrant SKP1 expression may be a pathogenic driver of cancer.

Collectively, the above data gleaned from a diverse array of patient-based genomic, transcriptomic and protein datasets show that *SKP1* is frequently misexpressed in human cancers, which

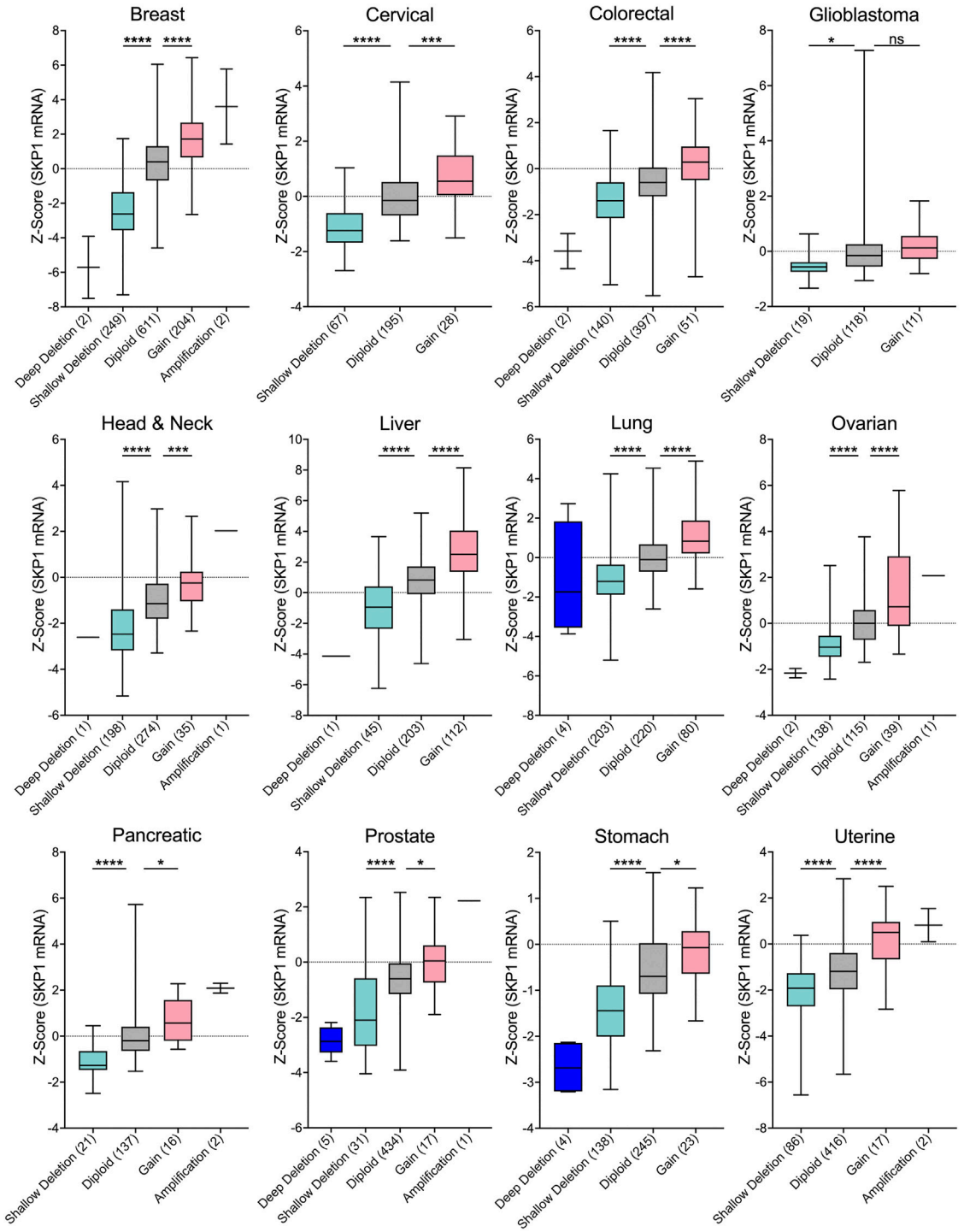

FIGURE 3 | *SKP1* Copy Number Alterations are Positively Correlated with mRNA Expression Levels in Cancer. Box-and-whisker plots of TCGA pan-cancer data from 12 common cancer types reveal linear correlations between *SKP1* copy number alterations and mRNA expression levels (Cerami et al., 2012; Gao et al., 2013; Hoadley et al., 2018). Boxes identify interquartile ranges (25th, 50th, and 75th percentiles), while whiskers depict entire range. For orientation purposes, the dotted horizontal lines identify 0. Specific copy number alterations (deep deletion; shallow deletion; gain; amp) and diploid categories are presented along the *x*-axis with the total number of samples indicated within brackets. Unpaired t-tests were conducted comparing either Shallow Deletions or Gains with the corresponding Diploid control (ns [not significant] *p*-value >0.05; * *p*-value <0.05; *** *p*-value <0.001; **** *p*-value <0.0001). Note that in general, very few deep deletions or amplifications were identified in the 12 cancer types.

suggests aberrant SKP1 expression may harbor tumor suppressive or oncogenic functions depending on whether it is under- or over-expressed, respectively. These apparently opposing activities may simply reflect that as a core SCF complex member, SKP1 may function as a tumor suppressor or oncoprotein depending on the protein targeted for degradation suggesting SKP1 expression levels may need to be precisely regulated to maintain cellular homeostasis, preserve genome stability and prevent cancer development and progression. Thus, the patient-based findings presented above underscore the need for additional insight into SKP1, its protein targets and the underlying biological mechanisms and their potential impact for oncogenesis. In this regard, future studies should also assess the clinical utility of *SKP1* as a potential prognostic indicator or a novel therapeutic target for cancers.

SKP1 AND THE SCF COMPLEX AS POTENTIAL THERAPEUTIC TARGETS IN CANCER

As the SCF complex regulates a diverse array of substrates involved in many biological pathways fundamental to genome stability, therapeutically targeting a core SCF component such as SKP1 may seem counter intuitive as there is the potential for increased toxicity and side effects. However, therapeutic success has been achieved with general proteasome inhibitors (e.g., Bortezomib (Robak et al., 2015)) and indirect SCF inhibitors (e.g., MLN4924 (Swords et al., 2015)) for the treatment of lymphoma, myeloma and leukemia lending support to use of broad-spectrum inhibitors targeting SKP1 and/or the SCF complex (Skaar et al., 2014). In fact, evidence shows cancer cells with a misregulated UPS are more sensitive to the broad-spectrum proteasome/SCF-targeting inhibitors than non-cancerous cells, which allows for the use of lower drug concentrations for effective outcomes and reduced side effects (Ludwig et al., 2005). Based on these findings, SKP1-targeted therapies designed to block SCF complex formation and function may represent promising treatment options. Rather than inhibiting global proteasomal degradation with agents like Bortezomib, or inactivating additional off-target Cullin family members with MLN4924, SKP1 inhibitors would specifically target the SCF complex, thereby reducing toxicity and ideally enhancing the therapeutic window (Silverman et al., 2012). Although a clinically administered dose would need to be strictly monitored, SKP1/SCF complex inhibitors could potentially be utilized in combination regimens with other chemotherapies to improve efficacy and/or help reduce the risk of drug resistance. For example, 5-fluorouracil, oxaliplatin, and irinotecan are first-line chemotherapies that induce DNA damage and cellular apoptosis (Longley et al., 2003). These drugs are often administered in combination for the treatment of colorectal cancer, with response rates from 40–50% and improved median survival (Douillard et al., 2000; Giacchetti et al., 2000; Longley et al., 2003). As the SCF complex is critical for eliciting an effective DNA damage response, perhaps co-treatment with a low-dose SKP1/SCF complex inhibitor would further sensitize cancer cells and synergize with standard chemotherapies to improve response rates and patient outcomes.

Considering the frequency of *SKP1* copy number losses in cancer (**Figure 2C**), it remains plausible that a synthetic lethal (SL) paradigm may prove highly effective in a broad range of cancer types. Synthetic lethality is defined as a rare and lethal genetic interaction occurring between two unlinked genes. In practice, cells harboring a mutation in either gene alone remain viable, whereas the presence of both mutations within a single cell will induce lethality (Sajesh et al., 2013). Although a relatively new therapeutic concept, SL strategies have already begun to enter the clinic as breast and ovarian cancers harboring *BRCA1/2* (Breast Cancer Type 1/2 Susceptibility Protein) defects are now being targeted with PARP1 (Poly [ADP-Ribose] Polymerase 1) inhibitors like Olaparib. Accordingly, genetic studies aimed at identifying SL interactors of *SKP1* are highly warranted as the SL interactors are candidate drug targets that when inhibited are predicted to induce the selective killing of cancer cells harboring *SKP1* defects. Beyond the genetic sensitization approaches detailed above, another promising strategy involves proteolysis-targeting chimeric molecules, or Protacs (reviewed in (Sakamoto et al., 2001; Burslem and Crews, 2020; Cecchini et al., 2021; Hughes et al., 2021)). The fundamental concept behind Protacs is that fusion proteins are created to link a specified target substrate to an F-box protein for SCF-mediated ubiquitination and degradation (Sakamoto et al., 2001). This approach would allow for conditional or tissue-specific degradation of overexpressed oncoproteins, suppression of tumor growth and cancer cell death.

AUTHOR CONTRIBUTIONS

Conceptualization LT, KR, CL, and KM; formal analyses, LT and KM; writing–original draft preparation LT, KR, CL, and KM; writing–review and editing, LT, KR, CL, and KM; supervision, KM; funding acquisition, KM. All authors have read and agreed to the published version of the manuscript.

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REVIEW
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The Tumor Suppressor Kinase LKB1: Metabolic Nexus

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Liver kinase B1 (LKB1) is a multitasking tumor suppressor kinase that is implicated in multiple malignancies such as lung, gastrointestinal, pancreatic, and breast. *LKB1* was first identified as the gene responsible for Peutz-Jeghers syndrome (PJS) characterized by hamartomatous polyps and oral mucotaneous pigmentation. LKB1 functions to activate AMP-activated protein kinase (AMPK) during energy stress to shift metabolic processes from active anabolic pathways to active catabolic pathways to generate ATP. Genetic loss or inactivation of *LKB1* promotes metabolic reprogramming and metabolic adaptations of cancer cells that fuel increased growth and division rates. As a result, *LKB1* loss is associated with increased aggressiveness and treatment options for patients with *LKB1* mutant tumors are limited. Recently, there has been new insights into the role LKB1 has on metabolic regulation and the identification of potential vulnerabilities in *LKB1* mutant tumors. In this review, we discuss the tumor suppressive role of *LKB1* and the impact *LKB1* loss has on metabolic reprograming in cancer cells, with a focus on lung cancer. We also discuss potential therapeutic avenues to treat malignancies associated with *LKB1* loss by targeting aberrant metabolic pathways associated with *LKB1* loss.

Keywords: LKB1, AMPK, mTOR, tumor suppressor, cancer metabolism, glycolysis, lung cancer

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INTRODUCTION

Metabolism is the outcome of key processes and reactions that generate energy to maintain cellular life. Metabolic processes serve to produce adenosine triphosphate (ATP) to meet the energetic demands of a cell, and the intermediates from these processes are used to generate biomolecules (Hanahan and Weinberg, 2011; Pavlova and Thompson, 2016). Metabolic reactions can either be anabolic (to buildup) or catabolic (to breakdown) and both these processes must be balanced to maintain the energy supply of cells while preserving biomolecules to sustain cellular function.

A common characteristic of cancer cells is an insatiable demand for energy in order to meet the needs for growth and proliferation. Cancer cells will take control over multiple signaling networks to reprogram metabolic pathways that enable cancer cells to synthesize biomolecules and adapt to survive under elevated reactive oxygen species (ROS) (Hanahan and Weinberg, 2011; Pavlova and Thompson, 2016). The mechanism behind metabolic reprogramming involves genetic adaptations through mutation of tumor suppressor genes and oncogenes that allow metabolic processes to be deregulated, leading to increased proliferation rate and survival of cancer cells. One such gene is the tumor suppressor serine/threonine kinase liver kinase B1 (*LKB1*), also known as serine-threonine kinase 11 (*STK11*).

LKB1 is implicated in multiple malignancies where it is often lost or inactivated. *LKB1* was first identified as the gene responsible for Peutz-Jeghers syndrome (PJS), a dominant disorder characterized by benign hamartomatous polyps in the gastrointestinal tract and mucocutaneous melanin pigmentation (Peutz, 1921; Jeghers et al., 1949; Hemminki et al., 1997). Germline mutations in *LKB1* that lead to the development of PJS result in loss of function of *LKB1* through truncations, deletions, or direct mutations to the kinase domain abolishing LKB1 kinase activity (Mehenni et al., 1998; Tiainen et al., 1999; Ylikorkala et al., 1999; Boudeau et al., 2003a). PJS patients have an increased risk of developing different malignancies, primarily in the gastrointestinal tract; colon, gastric, and intestinal cancers (Giardiello et al., 2000; Karuman et al., 2001), and are also susceptible to malignancies of the breast, lung, uterus, ovaries, cervix, and testes (Avizienyte et al., 1998; Nishioka et al., 1999; Boardman et al., 2000; Sanchez-Cespedes et al., 2002; Shen et al., 2002).

While *LKB1* mutations in PJS are associated with an increased risk of developing cancer, *LKB1* somatic mutations leading to malignancies are rare. It is surprising then, that the exception is in non-small cell lung cancer (NSCLC), where *LKB1* loss is implicated in 30% of cases (Sanchez-Cespedes, 2007; Ding et al., 2008; Gill et al., 2011). Furthermore, *LKB1* haploinsufficiency has been observed in the pancreas (Morton et al., 2010), breast (Shen et al., 2002), endometrial (Contreras et al., 2008) and liver adenocarcinoma (Kim and Chen, 2004) although very infrequent.

The tumor suppressor function of LKB1 has largely been attributed to the phosphorylation and activation of the energy sensor AMP-activated protein kinase (AMPK) in response to nutrient availability and energy stress. Here, the LKB1-AMPK axis shifts cellular metabolism from active anabolic pathways to active catabolic pathways to correct the energy imbalance (Hardie, 2005).

When LKB1 activity is abolished, the mechanism regulating metabolic pathways is eliminated. Loss of *LKB1* leads to increased glucose uptake and increased activity of aerobic glycolysis, commonly known as the Warburg effect (Warburg et al., 1927). Furthermore, loss of *LKB1* also leads to increased ROS that needs to be quenched to prevent damage to macromolecules. The survival of cancer cells relies on meeting the energy demand and adapting to the increased ROS produced. In this review, we discuss the tumor suppressive role of *LKB1* as a metabolic nexus, and how it is implicated in metabolic regulation, focusing on lung cancer. We also discuss the impact loss of *LKB1* has on metabolic reprograming and tumor progression and potential therapeutic avenues to treat *LKB1* deficient cancers by targeting aberrant metabolic pathways.

LKB1 MUTATIONS IN LUNG CANCER

LKB1 is spontaneously mutated most frequently in lung cancer patients is associated with increased aggressiveness (Calles et al., 2015; Lin et al., 2021). *LKB1* loss or inactivation is observed in 30% of lung adenocarcinoma. Lung adenocarcinoma (LUAD) is the most common type of lung cancer, accounting for 45% of cases. LUAD cases are stratified based on oncogenic mutation with ~60% of LUAD cases associated with *KRAS* and *EGFR* mutations (Sun et al., 2007; Collisson et al., 2014). *EGFR* mutations are more common in never smokers with the most frequently observed mutations being exon 19 deletions, and the point mutation codon 858 (L858R). *KRAS* oncogenic mutations are present in smokers and are often the result of base substitutions at codons 12 (91%), 13 (6%), and 61 (2%). *KRAS* codon 12 mutations result in amino acid substitutions of glutamine to either cysteine (G12C, 44%), valine (G12V, 23%), or aspartic acid (G12D, 17%) being the most common (Ostrem et al., 2013; Cox et al., 2014). KRAS is a small GTPase that promotes activation of the MAPK pathway to promote cell growth and survival (Burotto et al., 2014). Lung adenocarcinoma patients that present with *KRAS* mutations have a higher mutational burden and co-occurring mutations with tumor suppressors genes. Different co-occurring mutations with *KRAS* exhibit unique tumor behaviors and have different gene expression profiles (Skoulidis et al., 2015).

The most studied *Kras* lung cancer mouse model is the *LSL-Kras^{G12D}* mouse model. Using a transcriptional STOP element flanked by *loxP* sites, expression of *Kras^{G12D}* can be induced in multiple ways: tissue and cell-specific promoters driving Cre expression, inhalation of adeno-Cre (Ad5-CMV-Cre), or intratracheal administration of Ad5-CMV-Cre. With Ad5-CMV-Cre administration, lung cancer development can be followed in a time-dependent manner. Furthermore, the clonality of cancer development can also be studied. Expression of *Kras* in the lung caused characteristics of early adenocarcinoma, where lungs presented with atypical adenomatous hyperplasia (AAH), epithelial hyperplasia (EH), and adenomas. Although this mouse model recapitulated early disease histopathologies of lung cancer, it did not recapitulate late stages. When loss of function mutations of *Tp53* were combined with *Kras* oncogenic mutations, late-stage disease progression of lung adenocarcinoma was observed; nuclear atypia, elicit stromal desmoplasia, invasion, and metastasis (Jackson et al., 2005). It was not until *Lkb1* was co-mutated with *Kras* that lung adenocarcinoma disease progression exhibited a similar pattern and severity to human disease.

Mutations in other tumor suppressors (*RB1*, *CDKN2A*, *SMARCA4/BRG1*) are also frequently implicated in lung cancer. *TP53* is the most frequently mutated tumor suppressor in many cancers including lung cancer where it is mutated in ~70% of cases (Sanchez-Cespedes, 2007). *KRAS* mutant lung adenocarcinoma is often associated with a mutation in *CDKN2A* or *LKB1*. Mouse models of lung cancer typically use three different genotypes to recapitulate LUAD; *Kras;Tp53* (KP), *Kras;Cdkn2a* (KC), and *Kras;Lkb1* (KL). Each model exhibits different characteristics, severity, and aggressiveness of lung adenocarcinomas with different microenvironments, gene expression signatures, and responses to therapies (Skoulidis et al., 2015).

Early work on the KL mouse model characterized LUAD development compared to the KP genotype. The KL genotype is the only genetic combination to fully recapitulate human lung

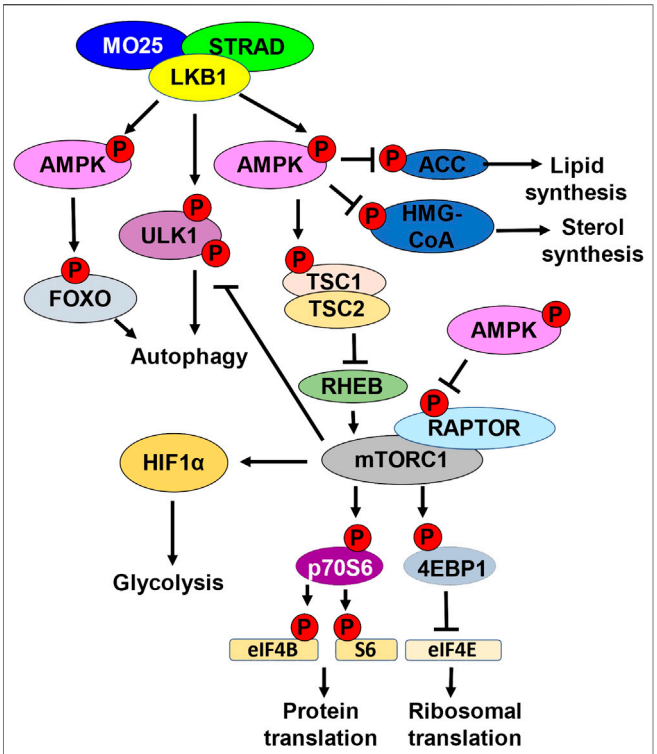

FIGURE 1 | LKB1 metabolic nexus. Schematic representation of downstream LKB1 signaling. LKB1 in complex with STRAD and MO25 phosphorylates and activates AMPK. AMPK phosphorylates and inhibits ACC, inhibiting lipid synthesis. AMPK phosphorylates and inhibits HMG-CoA, inhibiting sterol synthesis. Active AMPK also regulates autophagy by phosphorylating and activating FOXO. LKB1 can directly promote autophagy by phosphorylating ULK1. LKB1 activation of AMPK also inhibits mTORC1 kinase activation. mTORC1 kinase phosphorylates and activates p70S6. p70S6 phosphorylates and activates eIF4B and S6 kinase, promoting protein translation. mTORC1 also phosphorylates and inhibits 4EBP1, the inhibitor of eIF4E. eIF4e activation leads to increased ribosomal translation. mTORC1 can also inhibits autophagy by phosphorylating ULK1. mTORC1 promotes glycolysis by upregulating HIF1α. AMPK directly inhibits mTORC1 by phosphorylating RAPTOR. AMPK can also indirectly inhibit mTORC1 by phosphorylating and activating TSC1/TSC2. Active TSC1/2 complex inhibits RHEB.

adenocarcinoma in mice, showing all histological subgroups: squamous cell carcinoma, large cell carcinoma, adenocarcinoma, and adenosquamous carcinoma (Ji et al., 2007; Chong and Jänne, 2013; Shaw and Engelman, 2013). Adenosquamous and squamous subtypes are not seen in KP or KC models. *Lkb1* ectopic expression in KL, KC, or KP tumors significantly reduced growth and induced apoptosis, further demonstrating the functional classification of *Lkb1* as a tumor suppressor gene (Ji et al., 2007).

THE LKB1 KINASE

LKB1 is a conserved, ubiquitously expressed multitasking serine/threonine kinase with tumor suppressor function (Marignani et al., 2010). *LKB1* is a member of the Ca^{2+}-calmodulin dependent protein kinase family (Marignani, 2005) with orthologues in frogs, mice, worms, and flies (Smith et al., 1999; Watts et al., 2000; Martin and St Johnston, 2003). The human *LKB1* gene maps to chromosome 19p13.3 and is 23 kb long, consisting of 10 exons of which exons 1–9 are coding and exon 10 is non-coding. *LKB1* is transcribed in the telomere to centromere direction and generates a 50 kDa protein. *LKB1* is ubiquitously expressed in mice and humans with tissue-specific differences in overall abundance (Towler et al., 2008). In mice, Lkb1 protein is most abundant in embryonic and extra-embryonic tissues. Later in development, Lkb1 protein is concentrated in heart, esophagus, pancreas, kidney, colon, lung, small intestines, and stomach tissues (Luukko et al., 1999; Rowan et al., 1999). In adult mice, Lkb1 protein levels are most abundant in epithelial tissues, follicles, and corpus luteum of the ovary, seminiferous tubules of the testis, skeletal muscle monocytes, and glial cells (Rowan et al., 1999; Conde et al., 2007).

LKB1 is primarily found in a complex with the pseudokinase STE20-related adaptor (STRAD) (Baas et al., 2003) and the scaffolding protein Mouse protein 25 (MO25) (Boudeau et al., 2003b; Marignani et al., 2007). Unlike other kinases, LKB1 does not become catalytically active through T-loop threonine phosphorylation of the kinase domain, but instead when bound to adaptor proteins STRAD and MO25 (Baas et al., 2003; Boudeau et al., 2003b; Boudeau et al., 2006; Marignani et al., 2007; Dorfman and Macara, 2008). STRAD is a homolog of the STE20 family of kinases but lacks multiple critical residues required for a functional kinase domain (Dorfman and Macara, 2008). Although STRAD lacks a functional kinase domain, STRAD adopts an active conformation when bound to ATP (Zeqiraj et al., 2009). The binding of STRAD to MO25 enhances the affinity of STRAD to ATP. Furthermore, STRAD binding to ATP and MO25 are required for LKB1 catalytic activation (Baas et al., 2003; Boudeau et al., 2003b; Boudeau et al., 2006; Dorfman and Macara, 2008). LKB1 when in complex with STRAD and MO25 increases LKB1 catalytic activity by approximately 10 fold compared to LKB1 alone (Boudeau et al., 2003b) (**Figure 1**).

In addition to regulating the activity of LKB1, STRAD also regulates LKB1 subcellular localization, particularly nuclear-cytoplasmic localization. Individually, STRAD and MO25 can freely diffuse through nuclear pores and thus are localized both in the nucleus and cytoplasm. When *STRAD* and *MO25* are co-expressed, they exhibit exclusively cytoplasmic localization. LKB1 contains an N-terminal nuclear localization signal (NLS) directing LKB1 to the nucleus *via* an importin α/β mechanism. STRAD competes with importin α/β for binding to LKB1, and therefore binding of STRAD to LKB1 sequesters LKB1 in the cytoplasm. Co-expression of *LKB1* with *STRAD* and *MO25* causes LKB1 to localize in the cytoplasm (Baas et al., 2003; Boudeau et al., 2003b). STRAD promotes LKB1 nuclear export in a CRM1 and exportin7 dependent manner (Dorfman and Macara, 2008).

Oncogenic mutations generally occur in the kinase domain of *LKB1* and abolish kinase activity. *LKB1* introduction to cancer

cell lines that do not express *LKB1* results in a G1 arrest (Tiainen et al., 1999; Tiainen et al., 2002). Expression of catalytic deficient *LKB1* mutants, where the mutations are found within the kinase domain, does not result in a G1 arrest, suggesting that catalytic activity is required for tumor suppressor function (Scott et al., 2007). Furthermore, *LKB1* mutations that abolish the binding to STRAD-MO25 also do not exhibit cell cycle arrest functions suggesting that the binding of STRAD-MO25 to LKB1 is required for the cell cycle arrest function of LKB1 (Tiainen et al., 1999; Tiainen et al., 2002; Scott et al., 2007). Interestingly, the expression of *LKB1* with a catalytically deficient point mutation, $LKB1^{D194A}$, resulted in the expression of genes important in cell cycle progression (*CYCD1*, *RB1*, *CYCE*, and *CYCA*). This suggests that catalytic deficient mutants of LKB1 can promote cell growth through kinase-independent functions (Scott et al., 2007).

CHARACTERIZATION OF *LKB1* USING MOUSE MODELS

The early developmental link between *LKB1* and PJS motivated the generation of *Lkb1* loss of function alleles in mice to study the function of *Lkb1* in disease. Homozygous loss of *Lkb1* in mice resulted in embryonic lethality at midgestation. This lethality was attributed to abnormal *Vegf* regulation, where *Vegf* was expressed at higher levels compared to wild-type mice. *Vegf* expression is regulated in part through hypoxia-induced factor (Hif1α), suggesting that loss of *Lkb1* results in metabolic stress (Ylikorkala et al., 2001).

To better characterize Lkb1 function *in vivo*, conditional knockout alleles were generated to study the consequence of *Lkb1* loss in tissue specific manner. Conditional *Lkb1* knockout (KO) alleles were generated to induce *Lkb1* KO using Cre-recombinase (Bardeesy et al., 2002). Characterization of these alleles confirmed earlier observations that *Lkb1* loss is embryonic lethal and that *Lkb1* heterozygotes developed hamartomatous polyps like PJS patients (Bardeesy et al., 2002). Furthermore, DePinho's group determined that *Lkb1* heterozygous mice were more susceptible to carcinogenesis when exposed to 7,12-dimethylbenz(a)anthracene (DMBA) (Bardeesy et al., 2002).

This mouse model was used to identify the role of Lkb1 in energy metabolism. The Alessi group studied the effects of *Lkb1* KO in muscle tissues by inducing *Lkb1* KO using Cre driven under the *muscle creatine kinase (MCK)* promoter. Expression of *MCK-Cre* excised *Lkb1* in heart and skeletal muscle. They found that the AMP:ATP ratios were significantly elevated compared to control mice. This suggested that Lkb1 was involved in correcting the metabolic imbalance and that when Lkb1 activity is lost, muscle cells were not able to generate ATP to correct the imbalance (Sakamoto et al., 2005). This work led to the connection between LKB1 and energy metabolism. Later, it was discovered that LKB1 promotes the phosphorylation and activation of the AMPK family of kinases (Hawley et al., 2003; Lizcano et al., 2004).

LKB1 ACTIVATES THE AMPK FAMILY OF KINASES

LKB1 functions upstream of the AMPK of kinases family, which consists of AMPK, and 12 other related kinases termed the AMPK-related kinases (ARKs); novel (Nu) AMP related kinase 1 and 2 (NUAK1,2), salt inducible kinase 1–3 (SIK1-3), microtubule affinity regulating kinases 1–4 (MARK1-4), brain selective kinases 1 and 2 (BRSK1,2), and maternal embryonic leucine zipper kinase (MELK) (Scott et al., 2007).

Before the association of LKB1 with energy metabolism, the *C.elegans* ortholog of *LKB1* (*Par-4)* was implicated in cell polarity. Par-4 is asymmetrically localized during the first embryonic division, providing key signals for fate decisions in subsequent cell divisions (Watts et al., 2000). A role for LKB1 in regulating cell polarization was later observed in mammalian cells, suggesting evolutionary conserved function. Furthermore, the discovery that LKB1 phosphorylates and activates the ARK kinases in addition to AMPK provided insights into the potential mechanism behind the cell polarity related function of LKB1 (Lizcano et al., 2004). Both the MARK and BRSK kinases regulate microtubule dynamics and contribute to regulate cell polarity through LKB1 phosphorylation and activation (Kojima et al., 2007; Nakano and Takashima, 2012). MARK proteins phosphorylate and inhibit microtubule associated proteins (MAPs) causing microtubule depolymerization and reorganization (Kojima et al., 2007). A genetic screen in HEK293T cells identified the Hippo pathway protein YAP as a mediator of LKB1-MARK axis, implicating LKB1 in regulate organ size (Mohseni et al., 2014).

The tumor suppressor function of LKB1 is also partially mediated to the ARK kinases. LKB1 phosphorylation and activation of NUAK1 promotes cell cycle arrest in response to UV induced DNA damage. LKB1 and NUAK1 can phosphorylate cyclin-dependent kinase inhibitor 1A (CDKN1A), which inhibits cyclin-CDK complexes preventing G1/S transition and leads to a cell cycle arrest in a TP53 dependent mechanism (Zeng and Berger, 2006; Esteve-Puig et al., 2014). NUAK 1 and 2 are also implicated in glucose tolerance and attenuation of insulin signaling in muscle cells and regulate cell motility and muscle contraction through activation of myosin (Koh et al., 2010; Zagórska et al., 2010; Vallenius et al., 2011).

Finally, the LKB1-SIK axis plays a role in regulating metabolism. SIK phosphorylation of cAMP response element binding protein (CREB) and CREB regulated transcription co-activator (CRTC) regulates multiple biological processes including metabolism, cell differentiation, and proliferation (Gao et al., 2018). LKB1 activation of SIK kinases inhibits gluconeogenesis in liver cells, and the LKB1-SIK axis promotes growth and differentiation of adipose tissue (Patel et al., 2014; Darling and Cohen, 2021). Furthermore, LKB1-SIK axis can promote GLUT4 mediated glucose import in muscle and adipose tissues by phosphorylating and inhibiting CRTC2/3

(Stringer et al., 2015). SIK1 and SIK3 inhibit the expression of lipogenic genes, thereby reducing lipogenesis (Du et al., 2008; Sun et al., 2020).

The different and numerous ARK kinases highlight the diverse and cell specific functions of LKB1. LKB1 can directly phosphorylate and activate all members of the AMPK family except MELK. In this way, LKB1 acts as a master regulatory kinase acting through the AMPK family of kinases in a cell specific manner to regulate multiple pathways related to metabolism, cell polarity, migration, division, and transcription (Ylikorkala et al., 2001).

LKB1-AMPK IN METABOLIC REGULATION

The best characterized, and primary target of LKB1 to regulate energy metabolism is AMPK, which is the focus of this review. LKB1 functions upstream of AMPK, the central regulator in maintaining intracellular ATP levels. Upon activation under energy stress, when the AMP:ATP ratio is high, AMPK acts as a metabolic switch to inhibit anabolic (fatty acid and protein synthesis) pathways and promotes the activation of catabolic pathways (glycolysis, fatty acid oxidation, and autophagy) to correct the energy imbalance (Ciccarese et al., 2019). AMPK is a heterotrimeric protein kinase composed of a catalytic *a* subunit, and two regulatory subunits β and γ. AMP binds the γ subunit promoting a conformational change to remove allosteric inhibition of AMPK and promote its activation with additional Thr172 phosphorylation by upstream kinases. The primary kinase responsible for AMPK activation was identified to be LKB1, providing the first insights that the tumor suppressor function of LKB1 was mediated through AMPK to regulate energy metabolism (Hawley et al., 2003; Woods et al., 2003; Hardie and Alessi, 2013).

One of the direct readouts to assess AMPK activity is phosphorylation of acetyl-CoA carboxylase (ACC) which is the first enzyme in *de novo* lipid synthesis. AMPK-mediated phosphorylation of ACC is an inhibitory post-translational modification, thereby inhibiting fatty acid synthesis (Carling et al., 1987). AMPK also phosphorylates HMG-CoA reductase which plays a role in sterol synthesis (Sato et al., 1993). Fatty acid synthesis is important for cancer cell progression and *LKB1* mutant tumors exhibit elevated gene expression signature of genes involved in lipid synthesis (Carling et al., 1987; Bhatt et al., 2019) (**Figure 1**).

The LKB1-AMPK axis also inhibits the central integrator of energy and mitogenic signals to cell growth and division, the mammalian target of rapamycin complex 1 (mTORC1) (Corradetti et al., 2004). mTORC1 with its adaptor regulatory-associated protein of mTOR (RAPTOR), is a kinase responsible for promoting the phosphorylation of eukaryotic initiation factor 4E (EIF4E) binding protein (4EBP1), an inhibitor of EIF4E elongation factor, and p70 ribosomal S6 kinase 1 (p70S6). mTORC1 promotes the translation of mRNAs required for cell growth and division, including ribosomal proteins and proteins involved in translation (Thoreen et al., 2012) (**Figure 1**).

AMPK can indirectly inhibit mTORC1 by phosphorylating and activating tuberous sclerosis complex 2 (TSC2) (Corradetti et al., 2004). TSC2 functions as a heterodimer with TSC1, and together functions to indirectly inhibit mTOR through inhibition of the GTPase RAS homolog enriched in the brain (RHEB). When RHEB is active, RHEB binds and activates mTORC1 using its GTPase activity to induce a conformational change to the mTORC1 complex (Tee et al., 2003). TSC1/TSC2 bind and inhibit RHEB, thereby inhibiting mTORC1. AMPK can also directly inhibit mTORC1 by directly phosphorylating RAPTOR (Jewell et al., 2019). AMPK phosphorylation of RAPTOR promotes 14–3–3 protein binding, inhibiting mTORC1 from interacting with downstream targets 4EBP1 and p70S6 (Nojima et al., 2003) (**Figure 1**).

A consequence of *LKB1* loss is enhanced mTORC1 activation. mTORC1 promotes cell growth and metabolic changes such as increasing glycolysis and inhibiting autophagy. LKB1-AMPK directly, and indirectly through mTORC1, regulate autophagy. LKB1 directly phosphorylates ULK1 to promote autophagy and plays a role in mitochondrial biogenesis. mTORC1 phosphorylates and inhibits ULK1 inhibiting autophagy, and this is dysregulated in *LKB1* mutant cells. Furthermore, AMPK can promote the transcription of genes involved in autophagy through activation of the transcription factor FOXO (Kim et al., 2011) (**Figure 1**). Through the regulation of mTORC1, LKB1 and AMPK provide an important regulatory link between cell metabolism and cell growth and division.

LKB1 LOSS PROMOTES METABOLIC CHANGES

The LKB1-AMPK-mTORC1 axis is often deregulated in cancer cells, resulting in metabolic changes to support cancer cell growth and division. Cancer cells are often in a state of energy imbalance and have elevated energy requirements to manage high growth and division rates. Although producing less ATP, cancer cells show increased glycolysis rates even in the presence of oxygen. This shift to aerobic glycolysis demonstrates the Warburg effect (Pavlova and Thompson, 2016). LKB1 plays an important role in regulating glycolysis. Cells mutant for *LKB1* exhibit increased glucose uptake and increased extracellular acidification rate (ECAR). ECAR is determined by measuring lactate levels, which is produced by lactate dehydrogenase (LDH) to regenerate NAD+ for glycolysis. Lactate is then exported out of the cell causing acidification of the extracellular space. In the lung cancer cell line A549, which is deficient for *LKB1*, transient expression of *LKB1* resulted in a reduction of ECAR by 20% (Faubert et al., 2014). This suggests that LKB1 is an important regulator of glycolysis and that *LKB1* loss impacts glycolysis rate in cancer cells.

The metabolic reprogramming that occurs under *LKB1* loss is also observed in a Her2 positive *Lkb1* mutant breast cancer mouse model, $Stk11^{-/-}$/*NIC*. Loss of *Lkb1* reduces the latency of Her2 positive breast cancer from 197 to 147 days. Loss of *Lkb1* is associated with elevated mTORC1 activation and reduced AMPK activity (Andrade-Vieira et al., 2013). LKB1 acts as a metabolic

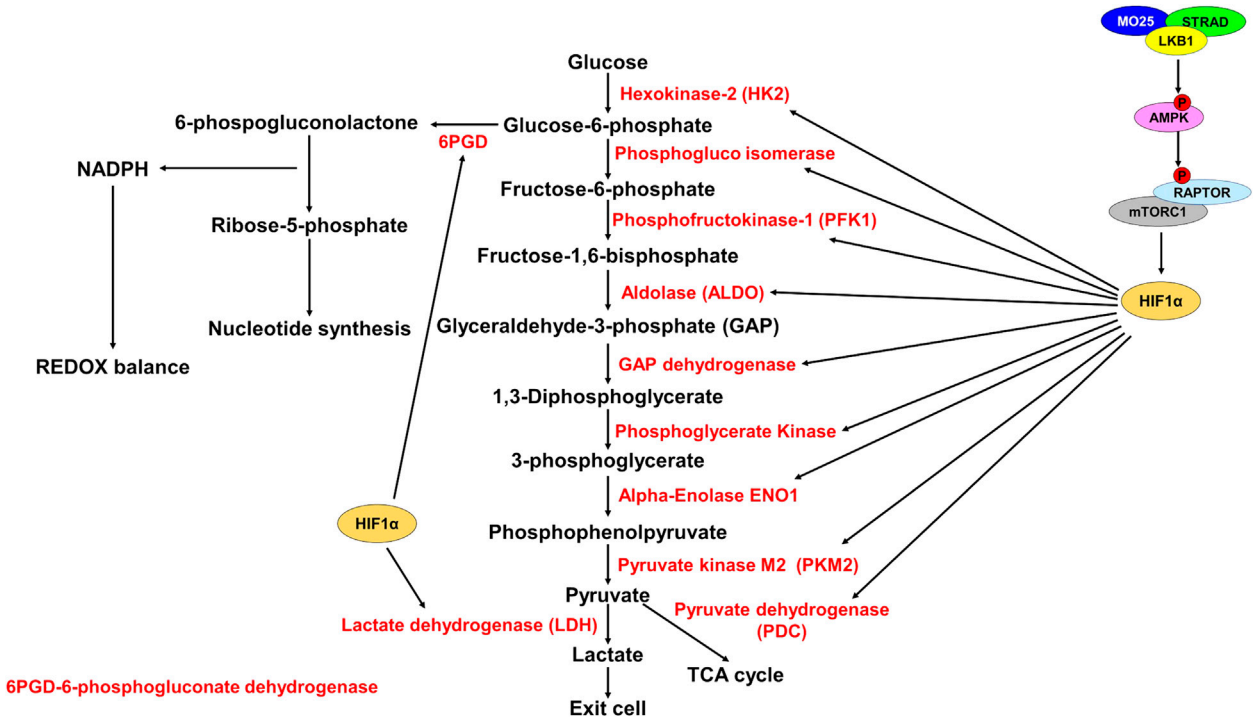

FIGURE 2 | HIF1α promotes expression of glycolytic enzymes. Schematic representation of glycolysis and the pentose phosphate pathway (PPP). LKB1 phosphorylates and activates AMPK which then phosphorylates and inhibits RAPTOR, thereby inhibiting mTORC1. mTORC1 promotes HIF1α upregulation. Active HIF1α promotes expression of glycolytic enzymes (red) increasing glycolysis rate. HIF1α also promotes expression of 6PGD to shunt glucose-6-phosphate to the PPP to generate NADPH for REDOX balance and for synthesis of ribose-5-phosphate for nucleotide synthesis.

nexus, connecting AMPK and mTORC1 signaling with downstream metabolic pathways. One such link is how *LKB1* loss impacts glycolysis. AMPK and mTORC1 are important regulators of glycolysis and since *Lkb1* acts upstream of AMPK and mTOR, the Marignani group examined the metabolic changes that occur when *LKB1* is lost. *Stk11*$^{-/-}$*/NIC* tumors exhibit increased branch chain amino acids (BCAA) valine and isoleucine and elevated glutamine and methionine levels and showed lower levels of glutathione, a ROS scavenger suggesting increased ROS stress which promotes tumor development. Furthermore, LDH and pyruvate dehydrogenase (PDH) levels are elevated, indicating elevated glycolysis (Andrade-Vieira et al., 2013).

In this model of breast cancer, loss of *Lkb1* synergizes with activated Her2, which promotes downstream activation of PI3K and AKT, leading to elevated mTORC1 activity. Elevated mTORC1 promotes metabolic reprogramming, increasing glycolysis by promoting the translation of glucose transporters, and glycolytic enzymes. The resulting increase in glycolysis is observed through elevated lactate (Andrade-Vieira et al., 2013) (**Figure 2**).

GLYCOLYSIS

Glycolysis is an important metabolic pathway that supports energy and biomolecule production, synthesis of ROS scavengers, and synthesis of nucleotides. Under normal physiological conditions, glucose is oxidized to pyruvate and shunted to the electron transport chain (ETC) for the generation of ATP by oxidative phosphorylation (OXPHOS). Under aerobic conditions, 1 molecule of glucose generates ~36 ATP and under anaerobic conditions, generates 2 molecules of ATP (Spinelli and Haigis, 2018). Glycolysis generates significantly fewer ATP molecules than OXPHOS but occurs at a much faster rate, therefore increased glycolysis can supply ATP production quicker under anaerobic conditions (Rogatzki et al., 2015). To do this, regeneration of NAD+ is required to support glycolysis. LDH generates NAD+ and lactate from pyruvate and NADH, thus supporting the increased glycolysis and ATP generation requirement (**Figure 2**).

The importance of glycolysis is not only through energy production, but glycolytic intermediates are important for many anabolic processes such as nucleotide synthesis and generating ROS scavengers (Lin et al., 2015; Ju et al., 2017). Cancer cells characteristically show increased utilization of glucose by glycolysis and a shift from OXPHOS to glycolysis to support their growth and division (Warburg et al., 1927). This idea has recently been questioned as OXPHOS was shown to still be utilized for energy production in some cancers (Davidson et al., 2016). OXPHOS is supported by lactate import and utilization through the TCA cycle, while glucose is used to support glycolysis and the pentose phosphate pathway (PPP) shunt to produce ROS scavenger NADPH. NADPH manages ROS stress and is important in generating ribose-5-phosphate for nucleotide biosynthesis (Lin et al., 2015; Ju et al., 2017) (**Figures 2,3**).

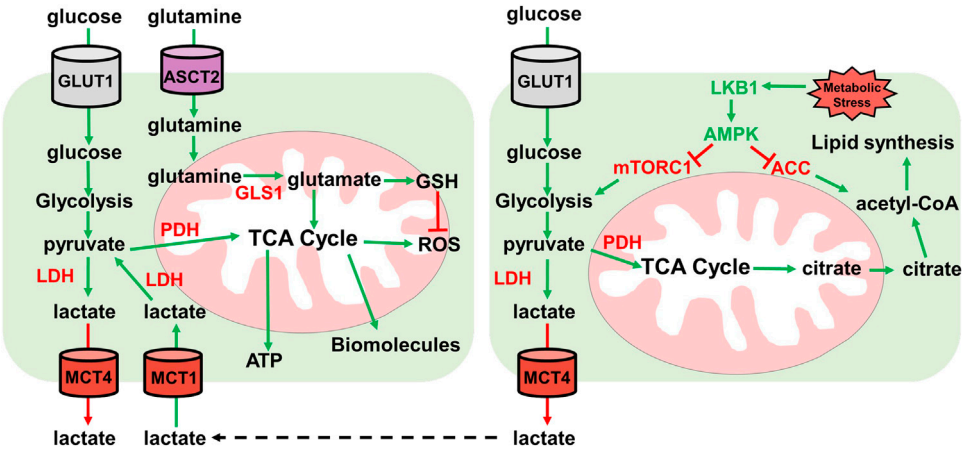

FIGURE 3 | Lactate and glutamine metabolism. Overview of lactate and glutamine metabolism. Glucose is imported by GLUT1 transporter. Glucose is then metabolized by glycolysis into pyruvate. Pyruvate then either enters the TCA cycle through pyruvate dehydrogenase (PDH) for ATP and biomolecule synthesis, or pyruvate is converted to lactate by lactate dehydrogenase (LDH). Lactate is then exported by MCT4 lactate exporter. Extracellular lactate can be imported by MCT1 and converted to pyruvate by lactate dehydrogenase for utilization in the TCA cycle for ATP and biomolecule synthesis. Glutamine is imported by ASCT2 where it then enters the mitochondria and is converted to glutamate. Glutamate can either enter the TCA cycle or is converted to GSH for ROS neutralization. Under metabolic stress conditions, the LKB1-AMPK pathway is activated. LKB1 activates AMPK which inhibits ACC and mTORC1. AMPK inhibition of ACC prevents lipid synthesis and mTORC1 inhibition by AMPK reduces glycolysis. Low glycolysis rate reduces TCA cycle generation of citrate which is used to generate acetyl-CoA for lipid synthesis.

LKB1 REGULATES GLYCOLYSIS

Elevated glycolysis rate is a hallmark of cancer, and tumors deficient in *LKB1* show elevated glycolysis rates, elevated glucose import, and increased expression of glycolytic enzymes. The effect *LKB1* loss has on glycolysis and glucose uptake is mediated by mTORC1 and concomitant oncogenic mutations. In *LKB1* mutant tumors, AMPK activity is diminished which leads to elevated mTORC1 activation (van Veelen et al., 2011). mTORC1 promotes the upregulation of the transcription factor HIF1α that becomes active under hypoxic conditions to promote aerobic respiration and ATP synthesis (Keith et al., 2012) (**Figure 2**).

HIF1α is regulated both at the protein level and translational level. Under normoxic physiological conditions, HIF1α is targeted for degradation by the E3 ligase von Hipple-Lindau tumor suppressor (Tanimoto et al., 2000). Under hypoxic conditions, HIF1α is stabilized where it translocates to the nucleus and induces gene transcription. Furthermore, mTORC1 promotes the upregulation of HIF1α. This was demonstrated in mouse embryonic fibroblasts (MEFs) by deleting *Tsc2* to promote mTORC1 activity. $Tsc2^{-/-}$ MEFs show elevated Hif1α levels, and this was dependent on mTORC1 targets 4Ebp1 and S6k1 (Düvel et al., 2010). In breast cancer cells, mTORC1 promotes HIF1α translation (Sakamoto et al., 2014). The increased mTORC1 activity also correlated with increased glucose uptake and utilization, which was suppressed by depleting *HIF1α*.

LKB1 mutant tumors show elevated *HIF1α* expression leading to increased glycolysis and lactate production (Andrade-Vieira et al., 2013; Faubert et al., 2014; Nam et al., 2016). Knockdown of *HIF1α* in *LKB1* mutant cells causes reduced growth and cell death under nutrient stress (Faubert et al., 2014; Nam et al., 2016). Gastrointestinal hamartomatous polyps from *Lkb1* heterozygous mice also show increased expression of the glucose transporter *Glut1*, and the first enzyme in glycolysis *hexokinase-2 (HK2)* (Shackelford et al., 2009). Both *GLUT1* and *HK2* are transcriptional targets of HIF1α (**Figure 2**).

Oncogenic mutations also synergize with *LKB1* loss to promote glycolysis and glucose uptake. *LKB1* is frequently concomitant with *KRAS* in lung cancers and *KRAS* oncogenic mutants upregulate glycolysis. KRAS was shown to promote *HIF1α* expression in colon cancer cells. Furthermore, Kerr et al. showed that *KRAS* copy number impacts glucose utilization (Kerr and Martins, 2018). Increasing the copy number of *KRAS* leads to increased glucose utilization, increased expression of *GLUT* transporters and glycolytic enzymes feeding the TCA cycle, and in later stages, glycolysis mediates management of ROS. Davidson et al. showed that in lung cancer models of $Kras^{G12D}$ and *Tp53* (KP mice), $Kras^{G12D}$ promotes increased glucose metabolism to generate pyruvate and lactate, consistent with observations that *Kras* oncogenic mutations yield elevated lactate (Davidson et al., 2016). Pyruvate is then shunted to the TCA cycle through pyruvate dehydrogenase (PDH), indicated by increased citrate levels. This indicates that in *Kras* models of lung cancer, glucose is utilized to generate both pyruvate and lactate, and pyruvate is shunted to the TCA cycle. *LKB1* loss can synergize with KRAS oncogenic mutations by increasing glucose uptake and glycolysis, leading to increased lactate production.

LACTATE AS AN ENERGY SOURCE IN LUNG TUMORS

Lactate has become more important in energy metabolism than once thought (Hui et al., 2017). Under normal metabolism where OXPHOS is functional, pyruvate is oxidized to acetyl-CoA by the pyruvate dehydrogenase complex (PDC). In hypoxic conditions,

pyruvate is converted to lactate by LDH. Cancer cells display elevated *LDH* expression, leading to elevated lactate formation and export. This creates an acidic tumor microenvironment which creates a favorable environment for invasion and metastasis and modulates immune cell functions (Rizwan et al., 2013; Xie et al., 2014; Brand et al., 2016). Furthermore, circulating lactate can be imported and utilized as an energy source and substrate for lipogenesis (Brooks, 2009; Chen et al., 2016). Lactate is imported using the lactate transporter monocarboxylate transporter 1 (MCT1). Inhibiting MCT1 reduces oxidative respiration and promotes an increase in glycolysis in both cancer cell lines in culture and xenograft models (Sonveaux et al., 2008; Pavlides et al., 2009) (**Figure 3**).

Lactate incorporation in tumor metabolism was seen in human NSCLC patients. NSCLC patients were infused with labeled glucose and incorporation of metabolic intermediates was measured from tumor samples. Results showed elevated lactate labeling compared to glycolytic metabolites (Faubert et al., 2017). Furthermore, *LKB1* mutant xenografts (H460 and HCC15 cells) in mice showed a similar phenotype with increased labeling of lactate (Faubert et al., 2017). In these xenograft models, infusion with labeled lactate showed labeled TCA intermediates, suggesting carbon from lactate can be incorporated into the TCA cycle (Faubert et al., 2017).

This study showed that in H460 and HCC15 cells, lactate shuttled in and out of the cells through lactate transporters. *MCT1* knockout HCC15 cells did not affect viability or division but displayed an increase in oxygen consumption rate (OCR) and decreased ECAR, indicating decreased glycolysis and decreased lactate production and export. This was also seen in HCC15 *MCT1* knockout xenograft models that when exposed to labeled lactate, showed reduced metabolite labeling of pyruvate and TCA intermediates. Like observations in cultured cells, *MCT1* knockout HC115 cell xenografts also displayed reduced glycolysis (Faubert et al., 2017).

This study illustrated the importance of lactate in *LKB1* mutant NSCLC tumors. Lactate can be utilized as an energy source through incorporation into the TCA cycle for ATP generation and TCA intermediates can act as precursors for synthesis of various amino acids. Extracellular lactate from neighboring cells can be imported and incorporated into the TCA through pyruvate (**Figure 3**). *LKB1* mutant lung cancer cells show elevated LDHA/B and the lactate transporter MCT1/4. The lactate export and import mechanisms highlight the uncoupling of glycolysis from the TCA cycle. Lactate cannot simply be incorporated into the TCA cycle, but rather extracellular lactate is imported for entry into the TCA cycle (Wu et al., 2016; Faubert et al., 2017).

LKB1 REGULATES ROS BALANCE: PPP AND GLUTAMINE METABOLISM

The metabolic reprogramming caused by loss of LKB1 activity results in elevated ROS and metabolic stress. Cellular metabolism generates reactive oxygen species that need to be quenched to prevent damage to DNA, proteins, and RNAs (Finkel, 2011). The increased aerobic glycolysis in cancer cells shunts metabolites towards the PPP to generate nucleotides *via* Ru-5-P and NADPH for ROS scavenging and lipid synthesis (Patra and Hay, 2014). NADPH is also an important mediator of glutathione ROS scavenging. Glutathione (GSH) and thioredoxins (TRX), two proteins that neutralize ROS, are synthesized using NADPH-dependent mechanisms (Nathan and Ding, 2010) (**Figure 3**). Depleting PPP genes causes increased ROS through defective ROS scavenging and leads to cancer cell death in colorectal cells (Ju et al., 2017). In lung cancer cell lines, depleting 6-phosphogluconate dehydrogenase (6PGD), decreases lipogenesis, RNA biosynthesis and increases ROS (Lin et al., 2015) (**Figure 2**). Furthermore, in A549 and H460 cells, both *LKB1* mutant cell lines, PPP-related genes are elevated indicating a reliance on the PPP pathway (Martín-Bernabé et al., 2014).

Glutamine is a common metabolite used in ROS scavenging and biosynthesis of biomolecules. Glutamine is essential for cancer cell growth in culture and is the most utilized amino acid. Glutamine can be shunted to the TCA cycle to provide acetyl-CoA or for the synthesis of other biomolecules. This is important in conditions where glucose metabolism generates lactate and not acetyl-CoA like in *LKB1* deficient tumors (Wise et al., 2011; Mullen et al., 2012). *KRAS* oncogene mutations synergize with *LKB1* loss to promote glutamine metabolism to combat ROS (Trachootham et al., 2006; Galan-Cobo et al., 2019). Glutamine can be used for amino acid and nucleic acid synthesis, further pointing to the essentiality for growth in cell culture (Caiola et al., 2016; Gwinn et al., 2018). In lung tumors *in vivo* however, glutamine does not enter the TCA cycle. GLS1, which is required for metabolizing glutamine in mitochondria, is not required for oncogenic *KRAS*-dependent growth (Davidson et al., 2016). As mentioned above, lactate is the primary carbon source for the TCA cycle (Faubert et al., 2017) (**Figure 3**).

Glutamine in *KRAS* oncogenic mutant tumors is necessary for mediating ROS neutralization. KL tumors are frequently concomitant for *KEAP2*, the inhibitor of oxidative stress response transcription factor NRF2. When *KEAP2* mutations are concomitant with KL mutations, accelerated tumor growth is observed (Romero et al., 2017; Galan-Cobo et al., 2019). Furthermore, when *LKB1* is expressed in *LKB1* deficient A549 cells, cell death is observed due to increased glutamine transformation and are less sensitive than control A549 cells to glutamine inhibitors (Galan-Cobo et al., 2019). *LKB1* mutant cells promote NRF2 dependent *GCL* expression, the primary enzyme to generate γ-Gly-Gly from glutamine and cysteine to increase GSH pools. Knockdown of *NRF2* in A549 cells causes decreased GSH formation from glutamine (Mitsuishi et al., 2012). The dependence of *LKB1* deficient cells to glutamine is also seen in polycystic kidney disease where *LKB1* mutant kidneys show increased dependence on glutamine to provide a synthesis of non-essential amino acids and GSH for ROS neutralization (Flowers et al., 2018).

HEXOSAMINE BIOSYNTHESIS

Another pathway that is elevated in *LKB1* mutant tumors is the hexosamine biosynthesis pathway (HBP). The HBP produces UDP-N-acetylglucosamine (UDP-GlcNAc) which is important for protein glycosylation. The HBP integrates intermediates from

glycolysis, lipid synthesis, glutamine metabolism, and nucleotide metabolism to generate UDP-GlcNAc (Vasconcelos-dos-Santos et al., 2015; Ferrer et al., 2016). UDP-GlcNAc is used by glycosyltransferases to generate glycoconjugates glycoproteins, glycolipids, and glycosaminoglycans. Glycosylation can modulate protein activity. For example, glycosylation inhibits phosphofructokinase 1 (PFK1) under hypoxic conditions, diverting glycolysis intermediates to PPP to decrease ROS (Sola-Penna et al., 2010).

Lung cancer cells have altered glycosylation and KL tumors display an elevated HBP gene expression profile. *LKB1* acts to suppress HBP, acting as an inhibitor of glycosylation. Inhibiting glycosylation by inhibiting glutamine-fructose-6-phosphate transaminase 2 (GFPT2) causes cell death in KL tumor cells but not in $KRAS^{G12D}$ tumor cells. This suggests a potential vulnerability in KL cells that can be exploited (Kim et al., 2020). KL mice treated with GFPT inhibitor Azaserine significantly suppressed tumor growth and this effect was specific to *LKB1* loss. This suggests a potential therapeutic target in KL lung cancers.

TARGETING *LKB1* MUTANT CANCERS THROUGH METABOLISM

The metabolic changes that occur under *LKB1* loss present an opportunity for therapeutic intervention by targeting aberrant metabolic pathways. *LKB1* mutant tumors display elevated glycolysis, lactate metabolism, and fatty acid synthesis due to the loss of AMPK activity, and subsequent loss of mTORC1 inhibition (Carling et al., 1987; van Veelen et al., 2011; Keith et al., 2012; Bhatt et al., 2019). Investigating downstream pathways could identify potential targets for therapeutic intervention. This is the case for biguanides metformin and closely related phenformin. Metformin is used to treat type II diabetes and functions by inhibiting mitochondrial complex I of the ETC (El-Mir et al., 2000; Dykens et al., 2008). The inhibition of complex I uncouples mitochondrial membrane potential, and reduces ATP generation through OXPHOS, leading to activation of AMPK in an LKB1 dependent mechanism (El-Mir et al., 2000; Dykens et al., 2008). In a mouse model of NSCLC where *Lkb1* is deficient, phenformin was able to reduce tumor growth (Shackelford et al., 2013). Although phenformin did not cause activation of AMPK, due to the lack of functional *Lkb1*, the disruption of OXPHOS and subsequent reduction in ATP caused metabolic stress and elevated ROS. *Lkb1* deficient tumors are unable to tolerate high levels of ROS and therefore undergo apoptosis as a result (Shackelford et al., 2013).

Cancer cells rely on elevated glycolysis and overexpress genes related to glucose metabolism (Hanahan and Weinberg, 2011; Pavlides et al., 2009). *LKB1* deficient tumors display elevated glycolysis signature mediated by elevated mTORC1 activity and *HIF1α* expression (**Figure 2**) (Andrade-Vieira et al., 2013; Faubert et al., 2014; Nam et al., 2016). Inhibitors against mTORC1 have limited therapeutic response in NSCLC. In NSCLC mouse models, the mTORC1 inhibitor rapamycin failed to induce a therapeutic response against KL tumors (Liang et al., 2010). mTORC1 inhibitors are often associated with resistance due to AKT-mTORC2 feedback loop, activating of mTORC1 (Wander et al., 2011). To circumvent potential resistance mechanisms associated with mTOR inhibitors, the Marignani' group conducted a pre-clinical trial that investigated strategies to inhibit energy and growth simultaneously by targeting glycolysis and mTOR as a potential therapeutic strategy for HER2 positive breast cancer. In $Stk11^{-/-}$/*NIC* mice, *Lkb1* loss synergizes with enhanced Her2 activation to promote activation of both arms of mTOR, mTORC1 and mTORC2, resulting in enhanced cell growth fueled by enhanced metabolism. They showed that simultaneously targeting glycolysis and mTOR complexes directly with glucose analog 2-deoxyglucose (2-DG) and mTOR inhibitor AZD8055 reduced tumor growth significantly and provided a stronger effect in combination than either 2-DG or AZD8055 alone (Andrade-Vieira et al., 2014).

Targeting glycolytic enzymes could provide a therapeutic window without dramatically affecting normal cells, which do not rely on elevated glucose metabolism (Pavlides et al., 2009; Hanahan and Weinberg, 2011). WZB117 is a glucose transporter inhibitor that showed promising results in NSCLC xenograft models using *LKB1* deficient A549 cells (Liu et al., 2012). WZB117 reduced ATP, GLUT1 and glycolytic enzyme protein levels (Liu et al., 2012). Furthermore, WZB117 significantly reduced tumor volume (Liu et al., 2012).

The hexokinase-2 inhibitor 2-DG has shown promising results in combination therapies (Maher et al., 2004; Andrade-Vieira et al., 2014). Hexokinase-2 catalyzes the phosphorylation of glucose, restricting its export and keeping glucose inside the cell (Bustamante and Pedersen, 1977; Bustamante et al., 1981). Paclitaxel and 2-DG significantly reduced tumor growth in NSCLC tumor xenograft model compared to single therapy alone (Maschek et al., 2004). By restricting the use of glucose by inhibiting hexokinase-2, other downstream metabolites are also reduced. Glycolysis intermediates cannot be shunted to the PPP to generate NADPH and Ru-5-BP for nucleotide synthesis restricting ROS scavenging and cell division (**Figures 2**,**3**). This will increase ROS stress, reduce tumor growth, and induce apoptosis.

LKB1 mutant tumors are susceptible to ROS stress and rely on multiple pathways to manage ROS stress. *LKB1* mutant tumors display elevated PPP pathway activity to generate NADPH along with increased glutamine metabolism to generate glutathione to scavenge ROS. Treating *LKB1* mutant tumors with compounds that increasing ROS or targeting KL tumors with glutamine inhibitors to reduce glutathione production inhibited tumor growth (Galan-Cobo et al., 2019). Glutamine metabolism is upregulated in KL tumors, where glutamine functions to generate glutathione to manage ROS stress (**Figure 3**) (Caiola et al., 2016; Davidson et al., 2016; Gwinn et al., 2018). Since *LKB1* deficient tumors are sensitive to increased ROS stress, compounds that produce excess ROS stress could serve as potential therapeutic agents for *LKB1* deficient tumors.

Another potential liability in *LKB1* mutant tumors is lactate metabolism. Similar to the findings by the Marignani group,

inhibition of glycolysis can also lead to reduced lactate production. Inhibitors of glycolytic enzymes or LDH can provide potential therapeutic avenues to explore. A549 cells deficient in *LDHA* showed reduced tumor formation after tail-vein injection (Xie et al., 2014). Inhibiting lactate production in KL tumors can restrain oxidative phosphorylation and amino acid synthesis by preventing TCA cycle regeneration. Furthermore, inhibiting lactate transporters could also be an effective approach. Knockout of *MCT1* in *LKB1* deficient cells reduced tumor growth in xenograft models, highlighting lactate metabolism as a potential therapeutic vulnerability (Faubert et al., 2017).

LKB1 mutant lung tumors display increased fatty acid deposits attributed to the elevated fatty acid synthesis pathways. Loss of AMPK activity causes ACC to become activated and promote fatty acid synthesis, while also impacting the expression of genes involved in fatty acid oxidation (**Figures 1**,**3**) (Carling et al., 1987; Bhatt et al., 2019). Targeting fatty acid synthesis by inhibiting ACC in mouse xenografts of *LKB1* deficient cells showed reduced tumor growth (Svensson et al., 2016). Lipids are important components in signaling molecules and for generation of membranes, and fatty acid synthesis is critical for cancer cell growth and survival. Targeting other genes involved in fatty acid synthesis provides other potential targets that could attenuate *LKB1* deficient tumor growth.

Exploiting metabolic vulnerabilities in *LKB1* deficient lung cancer provides promising avenues for the development of novel, effective therapies. Many metabolic pathways are regulated by LKB1. Understanding the impact of *LKB1* loss has on tumor metabolism provides a road map for identifying potential targets.

CONCLUSION

Metabolic adaptations by cancer cells are critical to meet the energetic needs and synthesis of macromolecules. Many cancer types show elevated glycolysis even in aerobic conditions. Glycolysis provides intermediates to support nucleotide synthesis and redox balance, both critical to supporting growth and division.

The tumor suppressor LKB1 plays an important role in regulating multiple metabolic pathways. By activating AMPK, LKB1 functions as a metabolic nexus, linking AMPK and mTORC1 signaling, to downstream metabolic pathways. *LKB1* loss is associated with multiple malignancies and causes metabolic reprograming to increase glycolysis and lactate production, elevate hexosamine biosynthesis and glutamine metabolism. Understanding metabolic pathways regulated by LKB1 can highlight therapeutic avenues by targeting pathways dysregulated due to *LKB1* loss and lead to better health outcomes in patients.

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MINI REVIEW
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Ubiquitin Binding Protein 2-Like (UBAP2L): is it so NICE After All?

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Ubiquitin Binding Protein 2-like (UBAP2L, also known as NICE-4) is a ubiquitin- and RNA-binding protein, highly conserved in metazoans. Despite its abundance, its functions have only recently started to be characterized. Several studies have demonstrated the crucial involvement of UBAP2L in various cellular processes such as cell cycle regulation, stem cell activity and stress-response signaling. In addition, UBAP2L has recently emerged as a master regulator of growth and proliferation in several human cancers, where it is suggested to display oncogenic properties. Given that this versatile protein is involved in the regulation of multiple and distinct cellular pathways, actively contributing to the maintenance of cell homeostasis and survival, UBAP2L might represent a good candidate for future therapeutic studies. In this review, we discuss the current knowledge and latest advances on elucidating UBAP2L cellular functions, with an aim to highlight the importance of targeting UBAP2L for future therapies.

Keywords: UBAP2L, mitosis, cancer, ubiquitin, stress signaling

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INTRODUCTION

Ubiquitin Associated Protein 2-Like (UBAP2L) or NICE-4 is a highly conserved protein in vertebrates (Chang et al., 2018). Encoded by the KIAA0144 gene located on the chromosomal region 1q21, NICE-4 was originally identified by Marenholz and colleagues in an effort to discover new Human Epidermal Differentiation Complex (EDC)-encoded genes (Marenholz et al., 2001). Five different isoforms produced by alternative splicing have been reported for UBAP2L, that are broadly expressed in nearly all tissues. Despite its abundant expression, UBAP2L has only recently attracted attention of broad scientific community which led to the discovery of its highly versatile roles. Interestingly, UBAP2L orthologs have been identified in metazoans such as Prion-like (Q/N-rich)-domain-bearing protein (PQN-59) in *Caenorhabditis elegans* and lingerer in *Drosophila melanogaster* (Uhlén et al., 2015).

UBAP2L is a 1,087 amino-acid (aa)-long protein, structurally composed of a N-terminal Ubiquitin-Associated Domain (UBA; aa 49-89), an Arginine-Glycine-Glycine (RGG; aa 131-190) domain and three predicted RNA-Binding regions (aa 239-257, aa 282-290 and aa 850-864) (Castello et al., 2016) (**Figure 1**). SILAC analysis demonstrated that UBAP2L cofractionates with ubiquitin in aggregates following proteasomal inhibition, emphasizing the functionality of its UBA domain (Wilde et al., 2011). Moreover, ribosome profiling studies demonstrated that UBAP2L promotes translation of target mRNAs suggesting that it can act as a ribosome-binding protein essential for protein synthesis (Luo et al., 2020). In addition, UBAP2L harbors a Domain of Unknown Function (DUF; aa 495-526). Prediction tools have unraveled several disordered regions prone to undergo Liquid-Liquid Phase Separation (LLPS) as well as several Nuclear Localization Signals (NLS) and Nuclear Export Signals (NES), suggesting that UBAP2L is shuttling between the cytoplasm and the

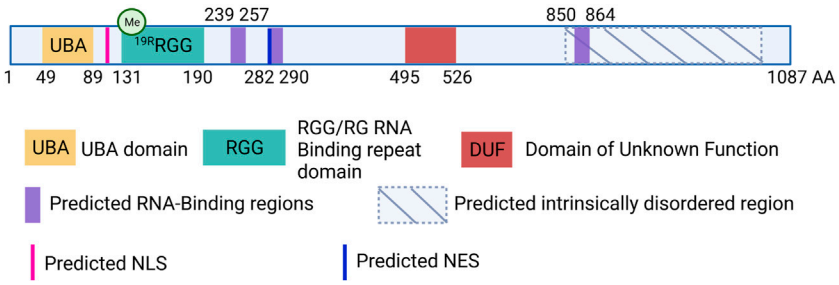

FIGURE 1 | Schematic view of the human UBAP2L protein and its domain organization. UBAP2L (1087 AA) is composed of a Ubiquitin-associated domain (UBA, yellow), an Arginine-Glycine-Glycine (RGG, green) domain and a Domain of Unknow Function (DUF, red). Additional RNA-binding regions have been predicted and are painted in purple. Moreover, UBAP2L is predicted to contain several Nuclear Localization Signals (NLS) and Nuclear Export Signals (NES) (pink and dark blue respectively). Several UBAP2L regions have been proposed to be intrinsically disordered (IDR), and prone to liquid-liquid phase-separation. The most conserved ones are shown with hatched lines. It is important to note that for simplicity we chose to depict only some of the predicted NLS, NES and IDR regions of UBAP2L in the scheme, and that this does not exclude the existence of other similar motifs or regions. Similarly, documented methylation modification on 19 different arginines (19R) present within the RGG domain has been indicated schematically.

nucleus. Such atypical domain organization classifies UBAP2L in both Ubiquitin-binding and RNA-binding proteins superfamilies, highlighting its potential involvement in a plethora of cellular processes.

Although UBAP2L was initially described as an interactor of the Human Zona Pellucida Sperm-binding protein 3 (ZP3) (Naz and Dhandapani, 2010), during the last decade additional studies have demonstrated its direct involvement in cell growth, mitotic progression, stem cell activity, apoptosis and stress response signaling (Bordeleau et al., 2014; Li and Huang, 2014; Chai et al., 2016; Maeda et al., 2016; Youn et al., 2018; Huang et al., 2020). Moreover, UBAP2L is overexpressed in different types of cancer, displaying oncogenic potential and often correlating with poor prognosis (Li and Huang, 2014; Zhao et al., 2015; Bai et al., 2016; Chai et al., 2016; Aucagne et al., 2017; He et al., 2018; Yoshida et al., 2020; Guan et al., 2021). Of note, UBAP2L KO mice die before birth or within minutes after surgical delivery from acute respiratory failure, demonstrating that UBAP2L holds housekeeping functions, essential for living organisms (Aucagne et al., 2017). This review discusses the current knowledge and the latest advances on elucidating NICE-4 cellular functions, with an aim to highlight the importance of targeting NICE4 for future therapies.

UBAP2L AND CELLULAR HOMEOSTASIS

UBAP2L and Stem Cell Activity

As mentioned above, UBAP2L KO mice die prematurely, pointing to a potential role for UBAP2L during development. Interestingly, in *C. elegans*, PQN-59 has been shown to modulate gene expression thus playing a key role in cell fate specification during development (Carlston et al., 2021). In an embryo, undifferentiated cells, called stem cells, give rise to one or several types of differentiated cells which later form mature tissues and organs. UBAP2L was proposed to be modified by O-Linked N-Acetylglucosamine (O-Glc-NAc) in mouse MC3T3E1 differentiating osteoblasts (Nagel et al., 2013). Interestingly, UBAP2L is found enriched in osteoblasts and as such it is used as an osteoblast marker (Guan et al., 2021). More globally, UBAP2L expression is increased in other types of undifferentiated cells such as mouse and human hematopoietic and leukemic stem cells. In the above study, Bordeleau and colleagues propose a model in which UBAP2L forms a complex with the Polycomb group (PcG) proteins BMI1 and Ring Finger Protein 2 (RNF2), thereby regulating long-term repopulating hematopoietic stem cells (LT-HSCs) independently of Ink4a/Arf locus repression, a popular target of BMI1. The authors suggest that at least two Polycomb-repressive complexes can assemble in order to regulate HSC function, which are distinguishable by the presence or the absence of UBAP2L (Bordeleau et al., 2014). Further investigations are needed in order to elucidate UBAP2L's precise role as part of the Polycomb complex since the exact mechanism has not been fully understood yet. A partial answer has been provided by Lin et al. who used rat bone marrow mesenchymal stem cells (BMSCs) overexpressing UBAP2L to transplant it to rats suffering from semi-sectioned spinal cord injury (SPI) and to monitor the recovery of the injured tissue (Lin et al., 2018). UBAP2L overexpressing cells exhibited stronger neuronal differentiation potential, which led to faster spinal cord function recovery. Mechanistically, UBAP2L overexpression results in increased expression of the cell cycle related protein cyclin D1 and of p38 MAPK, and more importantly to decreased expression of Caspase 3, a key apoptotic factor responsible for the majority of post-SCI neuronal death (Yu and Fehlings, 2011). Overall, the authors propose that UBAP2L overexpression in BMSCs promotes neuronal proliferation and survival, limits contingent damage like post-SCI inflammation and eventually leads to SCI repair (Lin et al., 2018). Given that the UBAP2L locus has been associated with other neuronal disorders such as bipolar or anorexia nervosa disorders (eQTLGen Consortium et al., 2019; Iranzo-Tatay et al., 2022), it would be of great interest to further investigate its potential role in the development of

other neurological and aging-related neurodegenerative diseases.

UBAP2L and Cell Division

In eukaryotes, mitosis is a crucial process which needs to be tightly regulated in time and space to allow for faithful division of a mother cell into two identical daughter cells (McIntosh, 2016). UBAP2L has been proposed to regulate cell division. Its depletion impairs chromosome alignment during metaphase and potentiates Spindle Assembly Checkpoint (SAC) response. Chromosome misalignment phenotypes upon UBAP2L depletion occur due to the disruption of stable k-fibers, suggesting defects in proper microtubule-kinetochore (MT-KT) attachment, which in turn hinders proper chromosome segregation and mitosis completion (Maeda et al., 2016). Maeda and colleagues further showed that UBAP2L RGG/RG domain is responsible for the multi- and micronucleation phenotypes observed in UBAP2L downregulated HeLa cells and more importantly that this function is mediated by the methylation of the arginines within the RGG/RG domain by the methyl-transferase PRMT1. Although the construct lacking this post-translational modification is properly localized at the spindle, it cannot rescue chromosome misalignment during metaphase observed in UBAP2L depleted cells suggesting that UBAP2L RGG/RG domain methylation is essential for proper MT-KT attachments, accurate chromosome distribution and proper mitotic progression. Consistently, UBAP2L depletion leads to an enrichment of G2/Mitotic (G2/M) population in HeLa cells (Maeda et al., 2016), in ZR-75-30 and in T-47D breast cancer cells (He et al., 2018) and in DU145 prostate cancer cells (Li and Huang, 2014) pointing to an important role of UBAP2L as a cell cycle regulator.

UBAP2L and Stress Signaling

An interesting feature of UBAP2L protein is its ability to aggregate and to regulate protein synthesis as indicated above (Wilde et al., 2011; Luo et al., 2020). mRNA turnover and protection under stress conditions have been associated with the formation of Stress Granules (SG) (Parker and Sheth, 2007). In an attempt to identify new components and/or regulators of cytosolic RNA granules, Youn and colleagues performed proximity-based proteomics and identified UBAP2L as a critical factor for efficient SG assembly following stress induced by the arsenite treatment. Importantly, the DUF domain of UBAP2L containing an phenylalanine-glycine phenylalanine-glycine (FG-FG) motif is critical for G3BP1 (Ras GTPase-activating protein-binding protein 1) recognition and binding in flies (Baumgartner et al., 2013) and is responsible for G3BP1 assembly in HeLa cells. In contrast, UBA and RGG domains of UBAP2L seem to be dispensable for SG formation (Youn et al., 2018). Subsequent studies by another group demonstrated the crucial role of the RGG domain of UBAP2L for SG competence under stress-null and stress conditions (Huang et al., 2020). More precisely, under stress conditions, UBAP2L methylation by PRMT1 is decreased, enabling UBAP2L's interaction with SG components and subsequently promoting SG assembly. The authors show that UBAP2L's DUF domain is still very important for G3BP1/2 NTF2-like domain binding and localization. In fact, depletion of the DUF domain promotes UBAP2L shuttling from the cytoplasm to the nucleus, impeding its interaction with G3BP1/2 and consecutively abolishes SG formation (Huang et al., 2020). Further work from Gotta group, propose that UBAP2L forms SG cores to which G3BP1 is subsequently recruited to allow for SG maturation, suggesting that UBAP2L acts upstream of G3BP1 in SG nucleation (Cirillo et al., 2020). Intriguingly, this phenomenon seems to be specific to human cells as a recent study from the same group established that PQN-59 and GTBP-1 (the human UBAP2L and G3BP1/2 orthologs respectively) are not essential for SG assembly in C. elegans (Abbatemarco et al., 2021). Interestingly, additional types of subcellular complexes can be assembled under stress conditions. Among them, the nuclear "twins" of SG are called paraspeckles (PS). These ribonucleoproteins (RNP) granules assemble around the long noncoding RNA (lncRNA) NEAT1 (Fox et al., 2018). Upon stress induction, SGs regulate PSs assembly via the sequestration of important negative regulators of PS formation such as UBAP2L (An et al., 2019). For the moment, we still lack sufficient knowledge to explain the molecular mechanism behind this regulation and it would be important to understand if and how UBAP2L acts as a global regulator of stress-induced complex assemblies, in addition to its well-established role in SGs.

UBAP2L AND CANCER

Recent work has demonstrated that UBAP2L is overexpressed in a variety of cancers and as such it has gained significant attention of researchers over the past years. Although its aberrant expression is a common feature of very different types of tumors, the way UBAP2L acts to promote carcinogenesis appears to be highly variable (**Figure 2**), highlighting UBAP2L's versatile functions not only in healthy tissues but also under pathological conditions. As mentioned above, UBAP2L is broadly expressed in almost all tissues. Likewise, this abundance is also found and exacerbated in distinct tumor types such as prostate, breast, uterine, cervical, non-small cell lung and gastric cancers, glioma, colorectal and hepatocellular carcinoma (HCC) and lung adenocarcinoma (Li and Huang, 2014; Zhao et al., 2015; Bai et al., 2016; Chai et al., 2016; Aucagne et al., 2017; Wang et al., 2017; Ye et al., 2017; He et al., 2018; Li et al., 2018; Lin et al., 2018; Pan et al., 2020; Yoshida et al., 2020; Guan et al., 2021; Yang et al., 2021; Li et al., 2022). In nearly all cited cancer studies, UBAP2L is suggested to act as an oncogene promoting cancer cell proliferation and growth *in vitro* and *in vivo*, thus providing an explanation to the existing negative correlation between UBAP2L expression and patients' prognosis.

UBAP2L Promotes Cell Proliferation and Growth

In prostate, breast cancers and HCC, UBAP2L depletion leads to an accumulation of G2/M cell population (Li and Huang, 2014; He et al., 2018; Li et al., 2018), whereas it was shown to increase

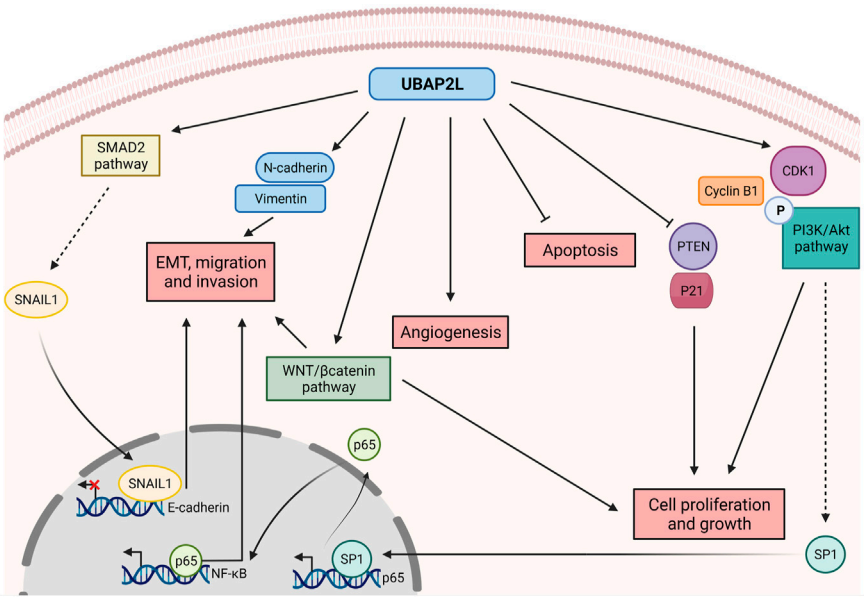

FIGURE 2 | Versatile roles of UBAP2L in promoting cancer disease. UBAP2L upregulates key cell cycle regulators such as CyclinB1, CDK1 and the PI3K/Akt pathway, while it inhibits the expression of tumor suppressors such as PTEN and P21, thereby promoting cell proliferation and growth. PI3K/Akt activation enhances SP1 levels which in turn activates P65 expression, thereby activating NF-κB pathway and favoring epithelial-mesenchymal transition (EMT), migration and invasion. The metastatic potential of UBAP2L-overexpressing cells is also sustained by the activation of the SMAD2 pathway, triggering the transcriptional repressor SNAIL1 to the E-cadherin promoter, shutting down its expression. Cancer cells overexpressing UBAP2L are characterized by hyperactivation of the WNT/βcatenin pathway and by upregulation of mesenchymal factors such as N-cadherin and Vimentin, resulting in increased invasion and proliferation. Finally, UBAP2L favors tumor vascularization while inhibiting cancer cells apoptosis. Overall, UBAP2L promotes cancer progression by regulating various axes of tumorigenesis known as the hallmarks of cancer.

the G0/G1 cells rate in Glioma and colorectal carcinoma, suggesting that UBAP2L may act during several cell cycle stages (Zhao et al., 2015; Chai et al., 2016). Additionally, UBAP2L is responsible for the multifaceted regulation of tumors' cellular and molecular properties in order to promote cellular survival as well as migration. Compelling evidence suggests that oncogenic pathways rely on the establishment of a suitable micro-environment that provides nutrients and supports tumor development and survival as elegantly summarized in 2011 (Hanahan and Weinberg, 2011). Intriguingly, UBAP2L seems to be involved in the regulation of several hallmarks of cancer.

Firstly, as mentioned above, UBAP2L sustains cell proliferation potentially via the regulation of cell cycle signaling pathways. For instance, it has been observed that knockdown of UBAP2L increases p21 and decreases CDK1 and CyclinB1 expression in breast cancer cells (He et al., 2018). This observation was further confirmed in HCC in a study showing a gene enrichment analysis after UBAP2L depletion. As previously demonstrated, the authors found PTEN and p21 among the most upregulated genes, while CDK1, CyclinB1, p-PI3K and p-AKT were among the most downregulated genes following UBAP2L silencing (Li et al., 2018). The signaling pathways downstream of PTEN, TP53 and PI3K/Akt are commonly dysregulated and hijacked in cancerous cells in order to promote their growth as extensively reviewed in the past years (Hollander et al., 2011; Khemlina et al., 2017; Levine, 2020). Of particular interest, the PI3K/Akt pathway is implicated in a broad range of cellular processes including cell proliferation but also apoptosis, angiogenesis, replicative immortality, invasion and metastasis, pointing out to UBAP2L oncogene as a golden target for future anti-cancer therapies (Lien et al., 2017). The molecular mechanism of how UBAP2L might regulate the PI3K/Akt pathway can be partially explained by a study suggesting that UBAP2L activates the PI3K/Akt pathway by promoting a phosphorylation cascade which in turn triggers SP1 binding to P65 promoter, inducing its expression. UBAP2L enables P65 translocation into the nucleus and possibly activates NF-KB (Li et al., 2022), a pathway strongly associated to cancer progression (Zinatizadeh et al., 2021). However, further efforts are required in order to dissect how UBAP2L precisely regulates signaling pathways to enable cancer progression.

UBAP2L Promotes Epithelial-Mesenchymal Transition, Migration, Invasion and Metastasis

An additional common feature of cancer cells is the ability to undergo epithelial-mesenchymal transition (EMT) as a means to promote effective invasion and metastasis (Hanahan and Weinberg, 2011). Interestingly, wound-healing assays of HCC cells lacking UBAP2L, revealed defects in migration and invasion. Consistently, cells lacking UBAP2L harbor increased epithelial (E-cadherin, CK-18) and decreased mesenchymal markers (N-cadherin, vimentin) (Ye et al., 2017), highlighting

UBAP2L's crucial role in regulating the metastatic potential of cancer cells. In addition to HCC, the promotion of EMT by UBAP2L has also been reported in prostate, lung and gastric cancers (Li and Huang, 2014; Aucagne et al., 2017; Lin et al., 2021). Complementary studies verified these conclusions *in vivo* where inhibition of UBAP2L led to defective cancer invasion in xenografts (Guan et al., 2021). In addition, mice injected with Ubap2l−/− A549 cells show less nodules in their lungs, lighter lungs and increased survival 3 weeks after injection in contrast to mice injected with Ubap2l+/+ A549 cells (Aucagne et al., 2017), while the opposite result is observed in gastric cancer when UBAP2L is overexpressed (Li et al., 2022). Finally, it was recently suggested that UBAP2L positively regulates the expression of the transcriptional repressor SNAIL1 *via* the SMAD2 signaling pathway which subsequently binds to and inhibits the promoter of E-cadherin, hindering the expression of this epithelial marker in favor of mesenchymal ones, ultimately leading to EMT, invasion and metastasis (Ye et al., 2017).

As previously discussed, cancer cells must use many diverse strategies to escape the cellular surveillance mechanisms in order to survive and migrate. To this end, most of the signaling pathways exploited by normal cells have to be hijacked, to favor cancer progression. For example, components of the Wnt/β-catenin signaling which is a highly conserved pathway regulating fundamental developmental processes, has been frequently observed to be mutated in cancer (Nusse and Clevers, 2017). Not surprisingly, UBAP2L has been proposed to activate the Wnt/β-catenin signaling cascade in gastric cancer cells, leading to the expression of downstream pathway targets, known to be implicated in tumorigenesis and metastasis (Yook et al., 2006; Liu et al., 2010; Damsky et al., 2011; Lin et al., 2021). However, the precise molecular mechanisms driving UBAP2L's oncogenic potential are not yet defined. UBAP2L has been reported as a BMI1 interactor as cited before (Bordeleau et al., 2014). Although BMI1 is essential for the activity of hematopoietic stem cells, it has also been suggested as a Wnt signaling activator by regulating the Wnt antagonist IDAX (Yu et al., 2018). Therefore, one hypothesis that could be further explored, might be that Wnt/β-catenin hyperactivity in UBAP2L-overexpressing tumors could be attributed to UBAP2L/BMI1 interaction.

UBAP2L Prevents Apoptosis of Cancer Cells and Promotes Tumor Vascularization

Cancer cells must acquire resistance to cellular death to ensure their survival and expansion (Hanahan and Weinberg, 2011). In this context, UBAP2L is suggested to act as an anti-apoptotic factor possibly by regulating, through yet unknown mechanisms, the expression of crucial apoptotic factors such as Bad/Bax and the cleavage of PARP and caspase 3 (Li and Huang, 2014; Chai et al., 2016). Bypassing all checkpoints employed by the cellular machinery is a challenge for cancer cells. Nevertheless, tumor microenvironment is crucial for proper cancer dissemination across tissues. For instance, cancerous cells require a certain amount of nutrients and oxygen to function properly and these components are efficiently brought to the cells only if the tumor is properly vascularized. Interestingly, samples from HCC patients revealed a positive correlation between UBAP2L and VEGF expression, a crucial protein for angiogenesis. Consistently, micro vessel density was also found to be increased in UBAP2L overexpressing tumors (Wang et al., 2017) and a complementary study from another laboratory reported that UBAP2L downregulation decreases the average vascular length and number of vascular branches (Li et al., 2018), once more pointing to a potential role for UBAP2L in favoring angiogenesis.

UBAP2L AND RNAS

Incremental studies were conducted on microRNAs (miRNAs), small nucleotides duplexes which post-transcriptionally regulate gene expression of their targets, being involved in general biological processes such as cell proliferation, apoptosis or brain development among others (Ambros, 2004). Intriguingly, UBAP2L was demonstrated to be targeted by different miRNA. First, in non-small cell lung cancer (NSCLC), miR-19a-3p directly inhibits UBAP2L, resulting in similar phenotypes as those observed upon UBAP2L downregulation, mainly inhibition of cell proliferation, migration and invasion (Pan et al., 2020). Similarly, UBAP2L was silenced by miR-148b-3p in gastric cancer cells leading to the same phenotypes as in NSCLC (Lin et al., 2021). Interestingly, the UBAP2L ortholog PQN-59 stabilizes several miRNAs involved in various cellular functions and interacts with RNA metabolism, transcription and translation cellular components similarly to UBAP2L, highlighting the importance of this protein in RNA regulation (Carlston et al., 2021). Supporting this hypothesis, UBAP2L localizes to stress granules and P-bodies under certain conditions, two structures highly linked to RNA turnover, miRNA or gene expression regulation (Leung et al., 2006).

Concluding Remarks

Conclusively, although UBAP2L has been identified more than 20 years ago, its extremely versatile roles in various signaling pathways have been elucidated only recently. It would therefore be fascinating that future studies address the underlying precise molecular mechanisms that govern and direct UBAP2L's functions towards such distinct signaling nodes to ensure cellular homeostasis. Our review aimed at highlighting the growing evidence on the oncogenic potential of UBAP2L that may identify UBAP2L as a promising target and stimulate research on UBAP2L-based future cancer therapies.

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Conceptualization, Writing, Review and Editing: LG, EP, and IS.

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Uncovering the spectrum of adult zebrafish neural stem cell cycle regulators

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Adult neural stem and progenitor cells (aNSPCs) persist lifelong in teleost models in diverse stem cell niches of the brain and spinal cord. Fish maintain developmental stem cell populations throughout life, including both neuro-epithelial cells (NECs) and radial-glial cells (RGCs). Within stem cell domains of the brain, RGCs persist in a cycling or quiescent state, whereas NECs continuously divide. Heterogeneous populations of RGCs also sit adjacent the central canal of the spinal cord, showing infrequent proliferative activity under homeostasis. With the rise of the zebrafish (*Danio rerio*) model to study adult neurogenesis and neuroregeneration in the central nervous system (CNS), it has become evident that aNSPC proliferation is regulated by a wealth of stimuli that may be coupled with biological function. Growing evidence suggests that aNSPCs are sensitive to environmental cues, social interactions, nutrient availability, and neurotrauma for example, and that distinct stem and progenitor cell populations alter their cell cycle activity accordingly. Such stimuli appear to act as triggers to either turn on normally dormant aNSPCs or modulate constitutive rates of niche-specific cell cycle behaviour. Defining the various forms of stimuli that influence RGC and NEC proliferation, and identifying the molecular regulators responsible, will strengthen our understanding of the connection between aNSPC activity and their biological significance. In this review, we aim to bring together the current state of knowledge on aNSPCs from studies investigating the zebrafish CNS, while highlighting emerging cell cycle regulators and outstanding questions that will help to advance this fascinating field of stem cell biology.

KEYWORDS

adult neural stem and progenitor cells, zebrafish, cell proliferation, cell cycle regulation, environmental enrichment, social behaviour, central nervous system repair, neurogenesis

Introduction

Teleost fishes serve as exceptional models to study the cell cycle dynamics and function of adult neural stem and progenitor cells (aNSPCs) throughout the central nervous system (CNS). The lifelong presence of proliferating aNSPCs across diverse stem cell niches of the brain (Zupanc et al., 2005; Grandel et al., 2006), along with their remarkable neuroregenerative capacity following brain and spinal cord injury (Zupanc and Sirbulescu, 2012) make teleosts extremely attractive to study. These attributes have

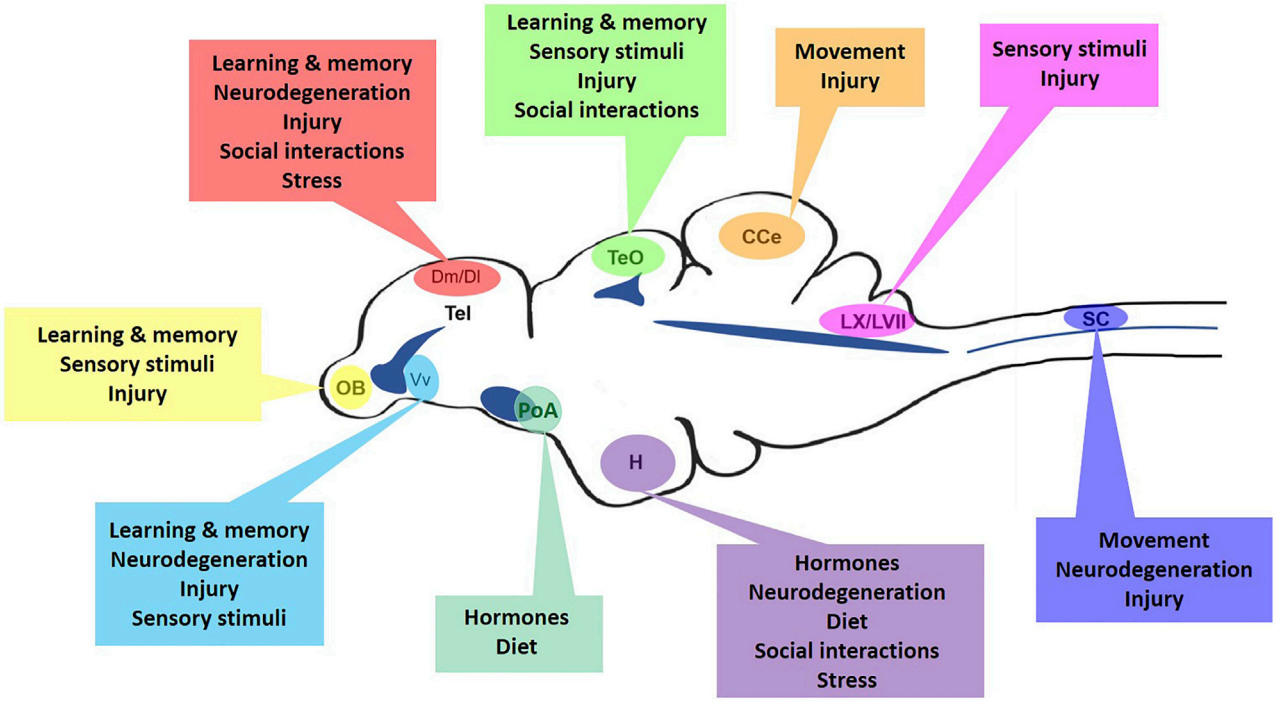

FIGURE 1
The many regulators of niche-specific aNSPCs in the adult zebrafish CNS. Mid-sagittal view of the zebrafish brain and spinal cord displaying major aNSPC niches and the array of homeostatic, injury-induced, or neurodegenerative regulators that may control their cell cycle dynamics. OB, olfactory bulbs; Tel, telencephalon; Dm, medial dorsal pallium; Dl, lateral dorsal pallium; Vv, ventral ventricular zone; TeO, optic tectum; PoA, preoptic area; H, hypothalamus; CCe, corpus cerebelli; LX/LVII, vagal and facial lobes; SC, spinal cord. Dark blue indicates approximate location of brain ventricles and the spinal cord central canal.

allowed researchers to take advantage of fish models to study the biological significance of adult neurogenesis (Lindsey and Tropepe, 2006) as well as the process of timely brain and spinal cord repair (Becker and Becker, 2008). Adult neurogenesis is defined as a lineage-directed process that commences with dividing aNSPCs that generate daughter cells fated towards a neuronal phenotype. A major difference between constitutive adult neurogenesis and regenerative neurogenesis that occurs after CNS damage, is that the latter largely relies on the activation of normally quiescent aNSPCs to re-enter the cell cycle. A fundamental question under homeostasis and following injury is what suite of factors are responsible for controlling aNSPC activity.

Distinguished as the most diverse vertebrate class and having adapted to nearly every aquatic environment (Kotrschal and Palzenberger, 1992), teleosts are excellent examples to explore how differences in habitat, environment, social interactions, and neurotrauma can impinge upon aNSPC function. The zebrafish (*Danio rerio*) has become one of the most popular laboratory models to study these factors in the brain and spinal cord. The social nature and complex behaviours displayed by this species (Kalueff et al., 2013), in combination with the multitude of molecular tools and small size of the CNS for imaging, has set the zebrafish apart to study neurogenesis and neurorepair. Unlike amniotes (Kriegstein and Alvarez-Buylla, 2009), fish retain developmental stem cell populations over ontogeny, including radial-glial cells (RGCs; aka ependymoglia in the spinal cord) in a quiescent or cycling state, and constitutively proliferating neuro-epithelial cells (NECs). These cells are distributed across diverse integrative and sensory niches of the brain and spinal cord (Lindsey et al., 2018). Therefore, this model offers the opportunity to uncover how unique biological contexts can stimulate aNSPC phenotypes (Figure 1), and help reveal the mechanisms regulating cell cycle activity.

Here, we provide an overview of the recent factors understood to regulate aNSPC cell cycle dynamics in teleosts, focusing on studies from the zebrafish model (summarized in Table 1). A key element of this review is to synthesize our knowledge of how day-to-day environmental stimuli can modulate constitutive rates of cell proliferation; an area poorly investigated in fish. We conclude by discussing key outstanding questions and available techniques to move these forward, to yield novel insight regarding the activity of aNSPCs under physiological and pathological conditions.

TABLE 1 Factors impacting aNSPC cell cycle activity in the zebrafish CNS.

Regulator	Stem cell niche	aNSPC	Δ cell cycle	References
Telencephalon				
[a]Estrogen	Whole brain	RGC & NEC	Decrease	Makantasi and Dermon, (2014)
Tank enrichment	Whole Tel	RGC & NEC	Increase	von Krogh et al. (2010)
Male social subordination	DP	RGC	Decrease	Tea et al. (2019)
Social stimulation	DP, PoA	RGC	Increase	Dunlap et al. (2021)
GnRH	PoA	RGC & NEC	Increase	Ceriani and Whitlock, (2021)
Dorsal stab lesion	Tel	RGC	Increase	Kroehne et al. (2011); Kishimoto et al. (2012); Barbosa et al. (2015)
cxcr5 knock-down	DP (injury)	RGC	Decrease	Kizil et al. (2012a)
Gata3 knock-down	DP (injury)	RGC	Decrease	Kizil et al. (2012b)
BMP/Id1	DP (homeostasis and injury)	RGC	Maintain quiescence	Rodriguez Viales et al. (2015), Zhang et al., 2021
Mdka	DP (homeostasis and injury)	RGC	Quiescence	Lübke et al. (2022)
Notch	DP (homeostasis and injury)	RGC	Quiescence	Chapouton et al. (2010); Anand and Mondal, (2017)
Inflammation	DP (homeostasis and injury)	RGC	Increase	Kyritsis et al. (2012)
Chronic hyperglycemia	Tel (homeostasis and injury)	RGC	Decrease	Dorsemans et al. (2017), Ghaddar et al. (2021)
Amyloid-β-42	Tel	RGC & NEC	Increase	Cosacak et al. (2019); Bhattarai et al. (2020)
Serotonin	Tel (AD model)	RGC	Decrease	Bhattarai et al. (2020)
BDNF	Tel (AD model)	RGC	Increase	Bhattarai et al. (2020)
IL-4/STAT6	Tel (AD model)	RGC	Increase	Bhattarai et al. (2020)
Serotonin promotion	PoA	RGC	Increase	Thompson et al. (2017)
Midbrain				
Visual deprivation	TeO	NEC	Decrease	Lindsey et al. (2014); Boulanger-Weill et al. (2017); Hall and Tropepe, (2018a)
Serotonin inhibition	H	RGC	Decrease	Pérez et al. (2013)
Serotonin promotion	H	RGC	Increase	Thompson et al. (2017)
Injury	TeO	RGC	Increase	Shimizu et al. (2018); Lindsey et al. (2019)
IL6/Stat3	TeO (injury)	RGC	Increase	Shimizu et al. (2021)
Injury	TeO	NEC	Increase	Shimizu et al. (2018); Lindsey et al. (2019)
Chronic hyperglycemia	MB	RGC & NEC	Decrease	Dorsemans et al. (2017)
Chronic starvation	TeO	NEC	Decrease	Benítez-Santana et al. (2016)
Corpus Cerebelli and Spinal Cord				
Injury	CCe	RGC & NEC	Increase	Kaslin et al. (2017)
Chronic hyperglycemia	CCe	RGC & NEC	Decrease	Stankiewicz et al. (2017)
Exercise	SC	RGC	Increase	Chang et al. (2021)
Injury	SC	RGC	Increase	Reimer et al. (2009)

[a]Brain-wide stem cell niches affected.

Only studies showing a significant effect on aNSPC proliferation are listed. Tel, Telencephalon; DP, dorsal pallium; PoA, preoptic area; AD, Alzheimer's Disease; TeO, optic tectum; H, hypothalamus; MB, midbrain; CCe, corpus cerebelli; SC, spinal cord; RGC, radial-glial cells; NEC, neuro-epithelial cells.

The effect of stress and social behaviour on adult neural stem and progenitor cell activity

Zebrafish are highly social species in the wild and under laboratory conditions (Suriyampola et al., 2016). Social interactions can take the form of predator-prey encounters, mating opportunities, conspecific relationships, and the formation of social hierarchies. This daily social plasticity commonly involves one or more chemosensory or visual cues, as well as the possibility of changes in swimming performance. This suggests that a broad range of adult niches could be implicated in aNSPC dynamics, including those processing sensory input and potentially even the spinal cord where additional motor neurons may be required to accommodate increased swimming. A consequence of these interactions is their effect on the physiology of the animal, such as the stress axis, and how this information acts to control aNSPCs behaviour.

In thinking how social behaviours modulate aNSPC activity, a key question is what constitutes a physiological stressful event.

Zebrafish RGCs are known to possess the glucocorticoid receptor Nr3c1 (Nelson et al., 2019), making them sensitive to changes in circulating cortisol. Higher cortisol levels have been identified in group-housed compared to individually-housed zebrafish (Onarheim et al., 2022), arguing against the idea that isolation consistently increases stress in social animals. One hypothesis may be that group composition, including sex ratios, fish size, and potential for hierarchies, are potential drivers of stress levels. Cues in the environment of isolated fish further appear to play a role in regulating cortisol levels. This has been illustrated by zebrafish having higher cortisol in an enriched-isolated context compared to a barren-isolated context (von Krogh et al., 2010). Work by Lindsey and Tropepe (2014) rather found that cortisol levels strongly correlated with social context, with the effect on aNSPC proliferation or neuronal differentiation being niche-specific. Social novelty and isolation revealed lower cortisol levels than grouped animals, with aNSPCs in sensory niches having the largest reduction in cell cycle activity. This work highlights the importance of pre-existing social experiences in shaping the future stress response and probability of stimulating niche-specific aNSPCs.

A small number of studies have also centered around the role of social hierarchies in driving aNSPC behaviour. One interesting report focused on sex-specific differences. In this study, the authors illustrated that subordinate males displayed reduced cell proliferation and neurogenesis in the dorsal telencephalon, along with increased cortisol levels (Tea et al., 2019). In females, however, no change was observed in dominant or subordinate animals when compared to group-housed controls. This suggests the possibility that hormonal differences may play a part in the cellular activity of aNSPCs. This finding aligns with earlier work studying socially suppressed subordinate male cichlids, where cell proliferation in the brain was lowest compared with dominant animals (Maruska et al., 2012). Notably, this study showed that if males were given the opportunity to rise in rank, the proportion of dividing aNSPCs increased in parallel. In the zebrafish, we now understand that changes in social status are closely correlated with gene expression patterns involved in neural plasticity in a niche-specific manner (Teles et al., 2016).

The emerging role of hormones and diet on adult neural stem and progenitor cell regulation

In the past few years, newer studies have emerged addressing the role of hormones and diet in regulating aNSPC activity in the zebrafish. This recent focus could provide valuable insight towards the importance of sex-specific differences, reproductive cycles, seasonality, food availability and nutrient composition in teleost fish. Several of these factors could also be increasingly important to consider during the design phase of experiments aimed at studying adult neurogenic plasticity.

Interestingly, in zebrafish only RGCs and not NECs are known to express the estrogen-synthesizing enzyme, aromatase-B (Pellegrini et al., 2016). Upon administration of estrogen to female zebrafish a reduction in cell proliferation in multiple brain regions has been reported, with the greatest impact in the dorsal/ventral telencephalon, preoptic area, hypothalamus, and cerebellum (Makantasi and Dermon, 2014). Within the niche of the preoptic area, animals treated with gonadotropin releasing hormone, but not testosterone produce an increase in cell division in non-RGC stem cell populations (Ceriani and Whitlock, 2021). Alternatively, inhibiting aNSPCs residing in the hypothalamus using the serotonin blocker tryptophan hydroxylase attenuates aNSPC proliferation, illustrating the dependency of stem cells in this domain on serotonin (Pérez et al., 2013).

How zebrafish diet or feeding regime relates to proliferative behaviour of aNSPCs is poorly characterized to date. Currently, studies have taken the form of either exposure to a high fat diet or starvation. Chronic hyperglycemia in adult zebrafish, for instance, was reported to impair homeostatic neurogenesis in the telencephalon, midbrain, and cerebellum, while also having a pro-inflammatory and oxidative stress effect (Dorsemans et al., 2017; Stankiewicz et al., 2017; Ghaddar et al., 2021). Conversely, 10 weeks of reduced food intake appears to be insufficient to alter aNSPC activity in the forebrain (Arslan-Ergul et al., 2016), but a 5 week starvation is adequate to decrease aNSPC proliferation in the optic tectum (Benítez-Santana et al., 2016). Additional niche-wide studies focusing on food type, nutrient composition, and feeding frequency undoubtedly would be beneficial to determine if distinct aNSPC populations are differentially affected and how this relates to changes in cell metabolism.

Sensory stimuli as a potent driver of adult neural stem and progenitor cell activity

Sensory input is a potent regulator of animal behaviour. In an aquatic environment teleosts receive this information from visual cues, olfactory and taste cues (i.e., chemosensory), as well as lateral line input (i.e., mechanosensory). A unique feature of zebrafish is that aNSPCs exist in neurogenic niches within primary processing sensory structures of the mature brain, including the forebrain olfactory bulbs (smell), midbrain optic tectum (vision), and hindbrain vagal/facial lobes (taste; Zupanc et al., 2005). These sensory domains offer the ability to study a range of modality-specific cues and their effect on aNSPC lineage activity. With several teleost models having conserved sensory niches, including the zebrafish, medaka, brown ghost knifefish, goldfish, and killifish (Ganz and Brand, 2016), this field is wide open to compare the functional role of aNSPCs using species-specific biologically relevant forms of sensory stimuli.

Studies applying forms of environmental enrichment or selective visual cues have shown a strong link between sensory input and aNSPC activity. A pointed example of how the mere opportunity for visual stimuli can induce changes in long-term aNSPC activity comes from recent work by Dunlap et al. (2021). Here, socially acclimated zebrafish that were first isolated, before being exposed to conspecifics in an adjacent tank chamber, was sufficient alone to enhance forebrain aNSPC activity. The above finding is supported by an early study exposing zebrafish to an enriched environment adorned with aquatic plants and gravel, or devoid of such items, showing a general increase in proliferation of forebrain aNSPCs with enrichment (von Krogh et al., 2010). An outstanding question is what effect does visual enrichment have on parent or progenitor NECs along the tectal marginal zone (Lindsey et al., 2018a), that would be predicted to be modulated.

Visual and chemosensory assays have also been employed to more directly test the effect of modality-specific sensory input on the activity of aNSPCs in corresponding sensory niches. Exposing zebrafish to monochromatic light has been demonstrated to decrease the proportion of cycling aNSPCs in the tectal sensory niche (Lindsey et al., 2014). Experiments using larval zebrafish also show that visual restriction impedes neurogenesis and functional integration into the optic tectum, correlating with reduced BDNF production (Boulanger-Weill et al., 2017; Hall and Tropepe, 2018a). In contrast, a 7-day treatment using a chemosensory assay resulted in an increase in neuronal survival but limited effect on RGC proliferation in the bulbs and vagal lobe (Lindsey et al., 2014). A major finding from this work was that modality-specific sensory input triggered the relevant sensory processing niche, and not those coding alternative modalities.

Taking advantage of sensory niches in the adult zebrafish brain further offers the chance to study the functional significance of aNSPCs for learning. Using sensory paradigms to explore the impact of task complexity on endogenous neurogenesis arising from sensory zones would provide new insight not possible in mammals. For instance, building on previous olfactory learning paradigms (Braubach et al., 2009), researchers can now ask how olfactory learning might modulate aNSPC activity along the Rostral Migratory Stream (RMS; Adolf et al., 2006; Kishimoto et al., 2012) as compared to resident RGC behaviour directly in the bulbs. This would provide valuable comparative data with mammals who maintain a lifelong RMS (Bond et al., 2015). In addition, since learning implicitly involved a memory component, with the dorsal lateral niche of the telencephalon homologous to the mammalian hippocampus (Ganz et al., 2015), the combined activity of aNSPCs in sensory and cognitive niches can be examined.

Modulation of spinal cord RGCs for the most part has not been explored as this population resides mainly in a quiescent state. Recently, one of the first studies of its kind has shown that zebrafish subjected to increased swimming using a swim tunnel was sufficient to enhance cell cycle proliferation of normally dormant spinal cord ependymoglia, and the generation of newborn motor neurons (Chang et al., 2021). Applying the same paradigm after spinal cord injury showed a similar trend, proposing that increased swimming exercise modulates neurogenesis. Conversely, experiments performing movement restraint in larval zebrafish have illustrated a decrease in neural stem and progenitor proliferation in the developing forebrain, though how robust this effect is across the neuro-axis remains unknown (Hall and Tropepe, 2018b). Together, these studies provide early support for the role of "exercise" in modulating baseline levels of aNSPCs in the brain and spinal cord of teleost models.

Cell intrinsic and injury-induced signals regulate adult neural stem and progenitor cells in the damaged central nervous system

Much effort has been placed on understanding the behaviour of RGCs and NECs following CNS damage compared to homeostatic modulation. More recently, this has also included examination of aNSPCs in a neurodegenerative context. Common to brain and spinal cord injury is the involvement of quiescent RGCs that are awakened following insult to re-enter the cell cycle and repopulate lost neuronal subtypes. In some instances, constitutively active NECs have been shown to additionally contribute to repair. Growing evidence suggests that aNSPCs retain diverse regenerative capacities that differ by stem cell niche in the adult CNS (Lindsey et al., 2018a), but their role in neurodegenerative diseases is only now beginning to be uncovered. This implies the need to better understand the balance between the factors comprising the damaged stem cell niche, and the intrinsic regenerative programs of distinct aNSPC populations. With many excellent reviews already published on this topic (Alunni and Bally-Cuif, 2016; Alunni et al., 2020), here we strive to provide a brief synopsis of the newest developments in this area to contrast with the regulators of aNSPCs observed under physiological conditions.

In the forebrain and spinal cord, RGCs drive the repair process. Accordingly, dorsal forebrain stab injury leads to the activation of RGCs, in addition to oligodendrocyte precursor cells (OPCs), that serve to replenish lost neurons and oligodendrocytes, respectively (Kroehne et al., 2011; Baumgart et al., 2012; Kishimoto et al., 2012; Barbosa et al., 2015; Sanchez-Gonzales et al., 2022). Most recently, transcriptomic analysis has provided insight regarding early proliferative signatures of RGCs and OPCs in the telencephalon and spinal cord after injury (Tsata et al., 2020; Demirci et al., 2022; Sanchez-Gonzales et al., 2022), adding to our existing knowledge of the role of *cxcr5* and Gata3 during neurorepair (Kizil et al., 2012a; 2012b). In the dorsal forebrain, genes regulating stem cell quiescence under

homeostasis, such as *mdka*, have also recently been shown to continue to be expressed following brain injury in non-reactive, RGC populations, suggesting a potential mechanism to prevent NSPC pool exhaustion (Lübke et al., 2022). This finding will add valuable information to our deep-rooted understanding of the role of Notch signalling in maintaining the quiescent state of NSPCs (Chapouton et al., 2010; Anand and Mondal, 2017). In the spinal cord, upon injury ependymoglia enter the cell cycle to produce motor neurons for functional swimming recovery (Reimer et al., 2009). In both the forebrain and spinal cord, numerous developmental regulatory pathways, such as Wnt, Notch, Shh, and ID1/BMP, are recapitulated following injury (Cardozo et al., 2017; Alunni et al., 2020; Zhang et al., 2021). Additionally, studies in the adult forebrain (Kyritsis et al., 2012) and larval spinal cord (Tsarouchas et al., 2018) confirm that activation of the immune response post-injury is critical to induce RGCs to regenerate. The rapid repair offered by the larval zebrafish following spinal cord injury has also gained traction as an efficient model for drug screening to identify therapeutics to test in mammals to improve spinal cord repair (Chapela et al., 2019; Nelson and Granato, 2022). Such studies equally afford the opportunity to examine how NSPC proliferative activity and intrinsic signaling pathways are modulated with application of these small molecules in an organism capable of successful spinal cord regeneration. Comparing the interaction between the immune response and RGCs or OPCs of the mature brain and spinal cord would be of great interest to identify CNS-wide themes in neuroregeneration. Unfortunately, detailed studies investigating the immune response following spinal cord injury in juvenile and adult stages are still lacking.

A small number of studies have also underscored the contribution of constitutively cycling NECs following brain injury. In the optic tectum, quiescent RGCs reside along the roof of the tectal ventricle whereas a pool of NECs sit at the midline tectal marginal zone, continuously furnishing the optic tectum with a small number of newborn neurons (Ito et al., 2010). Following tectal injury, it was reported that NECs amplify their cell cycle rates and neuronal output, while progeny of activated RGCs produce only newborn RGCs (Lindsey et al., 2019). Tectal injuries more proximal to the NEC population and in younger adult animals nevertheless showed evidence that RGCs could generate newborn neurons (Shimizu et al., 2018), suggesting that adult age appears to play a role in the regenerative potential of aNSPCs (Edelmann et al., 2013). This and more recent work has implicated Wnt and IL-6/STAT3 pathways in regulating RGC activity after injury in the tectum (Shimizu et al., 2018; 2021). In the adult cerebellum, NECs also appear to be the main contributor to restorative neurogenesis (Kaslin et al., 2017). However, in juvenile fish, RGCs of the cerebellum play a more prominent role in the regenerative process alongside NECs, giving rise to neuronal phenotypes similar to constitutive neurogenesis. Collectively, work on RGCs and NECs provide growing evidence that the regenerative process in zebrafish is accomplished by a combination of reactivated RGCs and constitutively proliferating NECs that are influenced by cellular senescence.

Models of Alzheimer's and Parkinson's disease have also been established in the zebrafish, permitting studies of aNSPC activity during the process of neurodegeneration. Alzheimer's models along with single-cell transcriptomics have illustrated neurodegenerative specific regulation of aNSPCs. These studies show that induced inflammation leads to a cascade of events, initiated by the activation of the IL4/STAT6 pathway. Subsequent downregulation of serotonin metabolism and promotion of BDNF in turn increase aNSPCs proliferation in a subtype and niche-specific manner (Bhattarai et al., 2016; Cosacak et al., 2019; Bhattarai et al., 2020). Parallel studies in older adult zebrafish showed similar levels of aNSPC proliferation, but a diminished immune response and fewer newborn neurons (Bhattarai et al., 2017). Modelling Parkinson's disease in the zebrafish, reports have shown that ablation of dopaminergic neurons leads to inflammation and RGC progenitor activity in the diencephalon to repopulate newborn dopaminergic neurons (Caldwell et al., 2019). Inducible transgenic lines have also come available to temporally ablate dopaminergic neurons (Godoy et al., 2015), providing a valuable tool to better interrogate this disease. While still new to the field of neurodegeneration, the zebrafish model offers many excellent advantages to elucidate the response of aNSPCs following Alzheimer's or Parkinson's disease that have yet to be capitalized upon.

Outstanding questions and future directions

The zebrafish is a tractable model to probe the mechanisms regulating aNSPC function. Still many outstanding questions persist. First, many niches of the brain are composed of populations of both quiescent and actively cycling RGCs. Adult neurogenesis by definition focuses on constitutively proliferating cells, whereas CNS regenerative studies concentrate primarily on the reactivation of quiescent aNSPCs. Whether certain forms of stimuli under homeostasis are sufficient to push quiescent RGCs into a cycling state, or a subpopulation of normally dividing RGCs of the forebrain might contribute to regeneration, is unclear. Equally unknown is whether RGCs or NECs can undergo shifts in their multipotency to generate specific neuronal lineages under different contexts, and if so, what are the intrinsic programs or changes in the microenvironment responsible for this cellular plasticity? Second, without knowledge of the molecular signature of similar aNSPCs found in different adult niches, it is unclear

whether their gene expression profiles align with their varied response to environmental or injury-induced cues. Many cell autonomous features also remain to be understood, including cell metabolism (Santoro, 2014), senescence (Da Silva-Álvarez et al., 2020), and the role of the unfolded protein response (Clark et al., 2020). Considering the stem cell niche, most of our knowledge is limited to the role of the immune response post-injury in activating aNSPC populations. Studying the involvement of neighboring cells, the extracellular matrix (Kim et al., 2018), and vasculature (Chen et al., 2019) is paramount to gain broader knowledge of niche-specific regulation of aNSPCs for successful neurorepair.

Methods to study the activity of zebrafish aNSPCs are extensive. These include the use of thymidine analogues such as BrdU and EdU, that label cells undergoing DNA synthesis (Cavanagh et al., 2011), as well as the endogenous cell cycle marker PCNA (Schönenberger et al., 2015). The dual Fucci line further allows changes in cell cycle phases to be fluorescently monitored (Bouldin and Kimelman, 2014); a generally underutilized tool thus far. More recent tamoxifen-inducible Cre-lox systems in zebrafish have allowed temporal colour-switching of aNSPCs to monitor population dynamics (Mosimann et al., 2011). Above all, the zebrabow system permits the most advanced multi-colour labeling to faithfully track stem cell lineages arising from parent stem cells (Pan et al., 2013). This suite of tools provides the opportunity to gain deeper insight towards aNSPC context-dependent activity. Combining these techniques with the multiple imaging approaches in the zebrafish, including live *in vivo* studies, 3-dimensional imaging of the CNS (Lindsey et al., 2018b), and advanced electron microscopy (Oorschot et al., 2021), the next decade promises to hold many exciting discoveries regarding teleost aNSPC activity, regulation, and biological significance.

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Conflict of interest

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REVIEW
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Embryonic Programs in Cancer and Metastasis—Insights From the Mammary Gland

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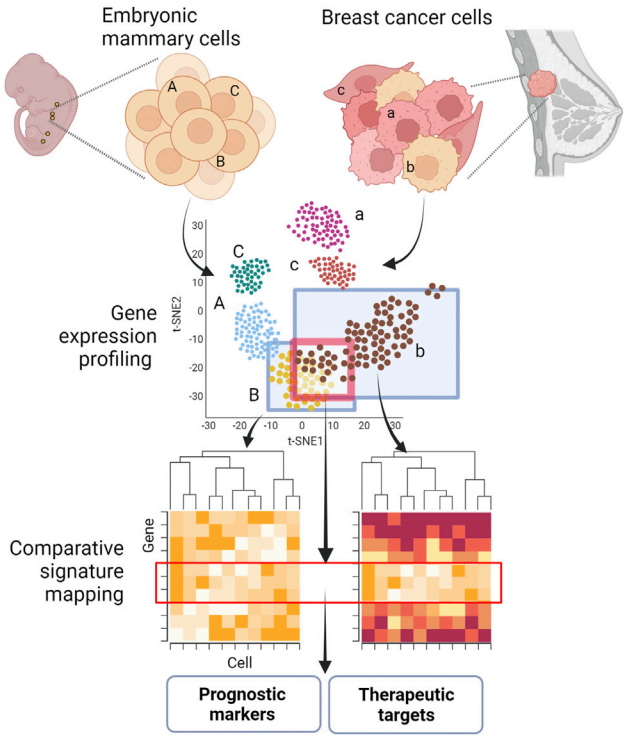
Cancer is characterized as a reversion of a differentiated cell to a primitive cell state that recapitulates, in many aspects, features of embryonic cells. This review explores the current knowledge of developmental mechanisms that are essential for embryonic mouse mammary gland development, with a particular focus on genes and signaling pathway components that are essential for the induction, morphogenesis, and lineage specification of the mammary gland. The roles of these same genes and signaling pathways in mammary gland or breast tumorigenesis and metastasis are then summarized. Strikingly, key embryonic developmental pathways are often reactivated or dysregulated during tumorigenesis and metastasis in processes such as aberrant proliferation, epithelial-to-mesenchymal transition (EMT), and stem cell potency which affects cellular lineage hierarchy. These observations are in line with findings from recent studies using lineage tracing as well as bulk- and single-cell transcriptomics that have uncovered features of embryonic cells in cancer and metastasis through the identification of cell types, cell states and characterisation of their dynamic changes. Given the many overlapping features and similarities of the molecular signatures of normal development and cancer, embryonic molecular signatures could be useful prognostic markers for cancer. In this way, the study of embryonic development will continue to complement the understanding of the mechanisms of cancer and aid in the discovery of novel therapeutic targets and strategies.

Keywords: mammary gland, embryonic development, breast cancer, metastasis, molecular signatures



Graphical Abstract | Shared molecular programs in embryonic mammary gland cells and breast cancers. Key developmental pathways are often reactivated or dysregulated during tumorigenesis and metastagenesis. The identification of embryonic signatures in tumors and metastasis could pinpoint novel prognostic markers and targets for therapeutic intervention. Figure created with BioRender.

INTRODUCTION

The mammary gland is the definitive feature of species in the class of Mammalia. Development of the mammary glands starts in the mouse embryo at embryonic (E) day 10.5 with the specification of the mammary line. By E11.75, all five mammary rudiment (MR) pairs in the mouse are present as disk-shaped, multi-layered placodal structures that will grow and acquire, sequentially, a morphology characterized by hillock, bud and bulb. Conventionally, the MR pairs are numbered 1 to 5 by their position along the antero-posterior axis. At E14.5, MR development diverges between the two sexes in a process called sexual dimorphism. MR development halts in males but proceeds in females with bulb enlargement and its recession into the mesenchyme. At E18.5, just before birth, the MR consists of a rudimentary ductal tree structure of 10–15 branches embedded within the mammary fat pad (Veltmaat et al., 2003; Spina and Cowin, 2021). The MRs develop asynchronously in the order of MR3, MR4, MR1 and MR5 and finally MR2, as determined by histological examination (Lee et al., 2011).

As a skin appendage, the murine embryonic mammary glands are excellent models for understanding developmental processes such as ectodermal specification, epithelial-mesenchymal cross-talks, morphogenesis, and their underlying cellular and molecular mechanisms. Various spontaneous mouse mutants and genetically engineered mouse models (GEMMs) have greatly facilitated the discovery of genes and signaling pathways that regulate mammary gland development (Veltmaat, 2017). This deeper understanding of developmental mechanisms has also proffered new perspectives regarding pathological conditions such as cancer. Indeed, the phases in embryonic mammary gland development resemble the phases of tumorigenesis and cancer progression. For example, the induction of the embryonic MR at around E11.5 and subsequent growth mediated by ectodermal cell recruitment (Lee et al., 2011) could be likened to cellular transformation and the development of carcinoma *in situ*. Further, MR sprouting at E15.5 and branching morphogenesis at E18.5 resemble the invasion of the basement membrane and tumor stroma during the metastatic cascade. Intriguingly, these embryonic morphogenetic events that mimic stages of tumorigenesis and metastasis also share similarities at the molecular level. Collectively, insight into these associations raises the possibility that cancer cells may leverage upon early developmental pathways and molecular programs to spur pathogenic development. Reversion to a more undifferentiated, embryonic-like state may promote processes that are associated with malignancy such as proliferation, epithelial-to-mesenchymal transition (EMT), cancer stem cell (CSC) formation, invasion, and metastasis (Kelleher et al., 2006; Takebe et al., 2011; Lee et al., 2019) (**Figure 1A**).

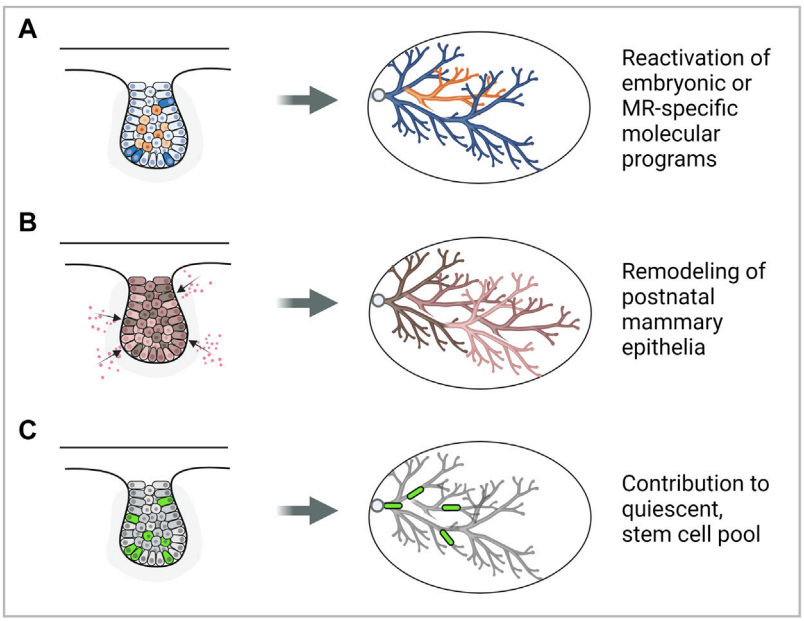


FIGURE 1 | Modes by which the embryonic mammary gland may contribute to breast cancer development. **(A)** Reactivation of embryonic mammary gland genes or signaling pathways may promote cancer development. **(B)** Exposure to carcinogens may remodel the postnatal mammary gland and increase breast cancer risk. **(C)** Embryonic mammary gland cells may contribute to the stem cell pool in the postnatal mammary gland which may be cells of origin of cancer. Note: a representative MR is depicted, however, each mode could be plausibly applied to MRs in other stages of development. Figure created with BioRender.

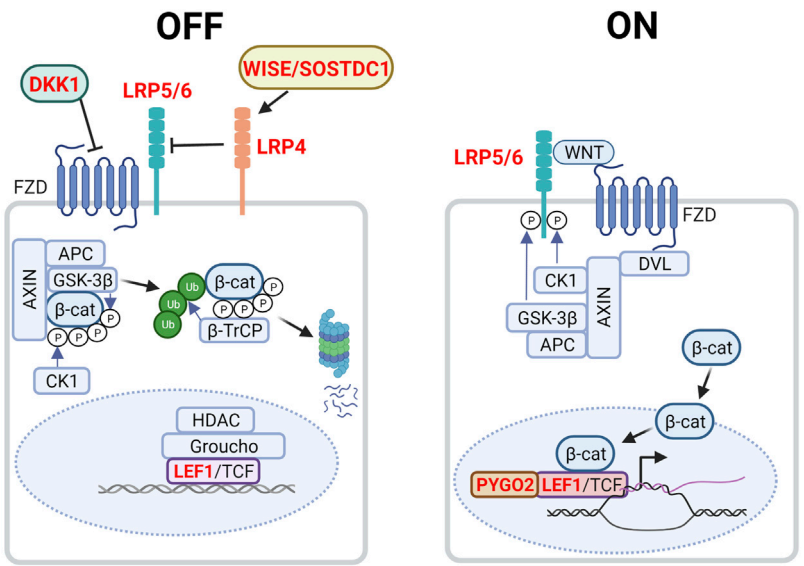


FIGURE 2 | Canonical WNT/β-catenin signaling in the embryonic mammary gland. In the absence of WNT (left, OFF state), cytoplasmic β-catenin is targeted to the destruction complex comprising AXIN, APC, GSK3β and CK1 where it is phosphorylated. Phosphorylated β-catenin is ubiquitinated by the E3 ubiquitin ligase β-TrCP, which targets β-catenin for proteasomal degradation. WNT target genes are repressed by Groucho and histone deacetylases (HDACs). LRP4 and its potential ligand, WISE/SOSTDC1 inhibits WNT signaling. In the presence of WNT ligand (right, ON state), a receptor complex forms between FZD and LRP5/6. DVL recruitment by FZD leads to LRP5/6 phosphorylation and AXIN recruitment. Consequently, degradation of β-catenin is disrupted, allowing β-catenin to accumulate in the nucleus where it functions as a co-activator of LEF1/TCF to promote the transcription of target genes. Genes highlighted in red denote those that give rise to aberrant embryonic mammary gland phenotypes when deleted or overexpressed. P denotes phosphorylation events after overexpressed. Figure created with BioRender.

Does embryonic developmental history affect postnatal development and susceptibility to cancer? Development of the MRs during embryogenesis may lay the foundation for growth and morphogenesis during the postnatal phase, which is subsequently mirrored during neoplastic transformation. It has been observed that the postnatal thoracic mammary glands (MGs

TABLE 1 | Genes and signaling pathways critical for embryonic mammary gland development, their corresponding role in breast cancer, involvement in other cancers, and available therapeutic targets and strategies.

Signaling pathway/ genes	Roles in embryonic mammary gland development	Roles in breast cancer	Other cancer types associated with gene/pathway dysregulation	Therapeutic targets/ strategies
WNT signaling *Dkk1* *Lef1* *Lrp4* *Lrp5/6* *Pygo* *Wise*	Induction Chu et al. (2004); Gu et al. (2009), morphogenesis Lindvall et al. (2006); Lindvall et al. (2009); Ahn et al. (2013), MR maintenance van Genderen et al. (1994); Boras-Granic et al. (2006), branching morphogenesis Lindvall et al. (2006); Gu et al. (2009); Lindvall et al. (2009)	Tumorigenesis Zhan et al. (2017), lineage specification, stem cell potency Centonze et al. (2020), metastasis Eyre et al. (2019)	Colorectal, gastrointestinal, leukemia, melanoma Zhan et al. (2017)	Anti-FZD antibody, small molecule inhibitors Wen et al. (2020)
HH signaling *Gli1* *Gli2* *Gli3*	Induction Hatsell and Cowin. (2006); Lee et al. (2011), morphogenesis Lee et al. (2011)	Tumorigenesis Fiaschi et al. (2009), EMT, development and maintenance of CSCs Tanaka et al. (2009); Zhu et al. (2019), invasiveness and metastasis O'Toole et al. (2011)	Basal cell carcinoma, medulloblastoma, pancreatic, colon, ovarian, and small-cell lung carcinomas Skoda et al. (2018)	Cyclopamine, SMO inhibitors, GLI1 antagonists (GANT58 and GANT61) Kubo et al. (2004); Bhateja et al. (2019); Riobo-Del Galdo et al. (2019)
FGF signaling *Fgf10* *Fgfr2b*	Induction Mailleux et al. (2002); Veltmaat et al. (2006), morphogenesis, MR maintenance, epithelial-mesenchymal crosstalk, branching morphogenesis Mailleux et al. (2002).	Cell proliferation McLeskey et al. (1998), metastasis McLeskey et al. (1998); Turner and Grose. (2010)	Lung, pancreatic, sarcoma Wiedlocha et al. (2021)	Tyrosine kinase inhibitors (TKIs), selective TKIs of FGFRs; monoclonal antibodies (mAbs) Santolla and Maggiolini. (2020)
P190B, IRS1, IRS2, IGF1R signaling	Induction, epithelial-mesenchymal cross talk and specification Heckman et al. (2007)	P190B—tumorigenesis, metastasis Heckman-Stoddard et al. (2009); McHenry et al. (2010), IRS1-metastasis suppressor, cancer stemness Ma et al. (2006), IRS2-metastasis promoter Nagle et al. (2004)	Esophageal, endometrial, ovarian, prostate, pancreatic Leitner et al. (2022)	IGF1R signaling inhibitors (NT compounds) IGF1R mAb, small molecule tyrosine kinase inhibitors (TKIs) of IGF1R and insulin receptor, and ligand neutralising strategies Ekyalongo and Yee. (2017)
PTHRP signaling	Mammary duct formation Wysolmerski et al. (1998), nipple sheath formation Foley et al. (2001), epithelial-mesenchymal cross-talk, sexual dimorphism Wysolmerski et al. (1998); Dunbar et al. (1999); Hiremath et al. (2012)	Cell proliferation, angiogenesis, apoptosis, bone metastasis Guise et al. (2002); Li et al. (2011)	Lung, prostate, colon, clear cell renal carcinoma, etc Edwards and Johnson. (2021)	PTHRP mAb Guise et al. (1996); Li et al. (2011)
BMP signaling *Bmp4* *Bmpr1a*	Mammary line positioning Cho et al. (2006), epithelial-mesenchymal crosstalk Hens et al. (2007), ductal outgrowth Hens et al. (2007)	Cell proliferation Alarmo and Kallioniemi. (2010); Zabkiewicz et al. (2017), EMT, cancer cells stemness, metastasis Huang et al. (2017), anoikis, negative regulator of metastasis Eckhardt et al. (2020)	Lung, adrenocortical carcinoma, medulloblastoma, colorectal, prostate, pancreatic, ovarian, bladder Bach et al. (2018)	Soluble decoy receptors, neutralising antibodies, BMPR kinase inhibitors Lowery and Rosen. (2018)
EDA signaling	Induction Mustonen et al. (2004), sexual dimorphism, branching morphogenesis Voutilainen et al. (2012)	Tumorigenesis and squamous metaplasia, pregnancy-dependent mammary tumors Williams et al. (2022)	Melanoma Vial et al. (2019)	N.A.
NRG3	Induction Howard et al. (2005), mammary mesenchyme specification Kogata et al. (2014)	Cell proliferation Hijazi et al. (1998)	Bladder, liver, lung, ovary, prostate, etc., Ocana et al. (2016)	N.A.
NOTCH signaling	Luminal cell specification and stem cell potency Lilja et al. (2018)	Oncogene Lamy et al. (2017); Krishna et al. (2019); Nandi and Chakrabarti. (2020), metastasis Mohammadi-Yeganeh et al. (2015), interactions with the tumor microenvironment Meurette and Mehlen. (2018)	Leukemia, adenoid cystic carcinoma, glomus tumor, lymphoma, squamous cell carcinoma, small cell lung carcinoma, urothelial carcinoma, esophageal, glioma Aster et al. (2017)	γ-secretase inhibitors, mAb, bispecific antibodies (anti-DLL4/VEGF), antibody-drug conjugates Lamy et al. (2017); Krishna et al. (2019)
HOX *Hoxc6* *Hoxd9* *Msx1* *Msx2* *Pax3*	Induction Veltmaat et al. (2006), morphogenesis Garcia-Gasca and Spyropoulos. (2000); Satokata et al. (2000), mammary mesenchyme formation Satokata et al. (2000)	Tumorigenesis Care et al. (1998); Briegel. (2006), tumor suppression Gilbert et al. (2010), metastasis Sun et al. (2013)	Leukemia, colorectal, liver, gastrointestinal, pancreatic, etc., Li et al. (2019)	HXR9 peptides Morgan et al. (2012)

(Continued on following page)

TABLE 1 | (*Continued*) Genes and signaling pathways critical for embryonic mammary gland development, their corresponding role in breast cancer, involvement in other cancers, and available therapeutic targets and strategies.

Signaling pathway/ genes	Roles in embryonic mammary gland development	Roles in breast cancer	Other cancer types associated with gene/pathway dysregulation	Therapeutic targets/ strategies
TBX *Tbx2* *Tbx3*	Mammary line positioning Cho et al. (2006), induction Davenport et al. (2003), MR maintenance Jerome-Majewska et al. (2005), nipple formation, branching morphogenesis Jerome-Majewska et al. (2005)	Tumorigenesis Yarosh et al. (2008), CSC formation Fillmore et al. (2010), EMT Wang et al. (2012), metastasis Rowley et al. (2004)	Pancreatic, colorectal, melanoma, endometrial, ovarian and cervical, rhabdomyosarcomas, ovarian etc Wansleben et al. (2014)	N.A.
GATA3	Induction, morphogenesis Asselin-Labat et al. (2007)	Tumor suppressor Asselin-Labat et al. (2011), oncogene Shan et al. (2014), luminal lineage differentiation, negative regulator of EMT Yan et al. (2010), negative regulator of metastasis Dydensborg et al. (2009); Yan et al. (2010)	Urothelial carcinomas, basal cell carcinoma, skin squamous cell carcinoma, salivary gland ductal carcinomas, pancreatic, etc Miettinen et al. (2014)	N.A.
P63	Induction (Mills et al., 1999; Yang et al., 1999)	EMT, cell motility, invasion (Lodillinsky et al., 2016; Yoh et al., 2016), stemness (Memmi et al., 2015)	Prostate, bladder, thyroid, lung, cervix (Melino, 2011)	N.A.
Hormone signaling	Sexual dimorphism	Tumorigenesis Manavathi et al. (2013), EMT, metastasis Saha Roy and Vadlamudi. (2012); Mohammadi Ghahhari et al. (2022), tumor microenvironment remodelling Bouris et al. (2015); Vella et al. (2020)	Ovarian, prostate, leukemia, lymphoma, lung, etc., Ahmad and Kumar. (2011)	Selective ER modulators (SERMs), selective ER down-regulators (SERDs), and steroidal or non-steroidal aromatase inhibitors (AIs) Siersbaek et al. (2018)

1, 2, and 3) tend to form mammary tumors more frequently than inguinal glands (MGs 4 and 5) (Sheldon et al., 1982; Minasian, 1983; Bolander, 1990). This has been attributed to the presence of more epithelial tissue in the thoracic glands compared to the inguinal glands that can undergo neoplastic transformation (Vaage, 1984). Moreover, the asynchronous differentiation between the thoracic and inguinal mammary glands results in increased less-differentiated structures such as the terminal end buds in the thoracic glands. This may explain the thoracic glands' increased susceptibility to 7,12-dimethylbenz(a)anthracene (DMBA)-induced carcinogenesis (Russo and Russo, 1988). These observations are in line with studies showing that the mouse mammary tumor virus long terminal repeat-driven Polyoma virus middle T antigen (MMTV- PyMT) and MMTV-cNeu mouse models generate more tumors in specific thoracic mammary glands than inguinal ones (Veltmaat et al., 2013) (**Figure 1B**). Lastly, embryonic mammary gland development is adversely affected by its exposure to endogenous and synthetic estrogens such as Bisphenol-A (BPA) which is linked to increased breast cancer risk during adulthood (Acevedo et al., 2013; Speroni et al., 2017) (**Figure 1B**). Taken together, physiological differences that are associated with differential tumorigenic potential could, in part, be attributed to differential molecular regulation that has taken place in embryonic development.

The approach of seeking to understand pathological conditions from the study of normal development has provided new perspectives into the origins of cancer. Some suggest that breast cancer may have a stem cell origin as the transcription factors that normally regulate gene expression in embryonic stem or progenitor cells are also misregulated in breast cancers (Briegel, 2006). Moreover, lineage tracing studies show that proliferating, long label-retaining embryonic cells (Boras-Granic et al., 2014) and a $Lgr5^{+}Tspan8^{hi}$ subpopulation (Fu et al., 2017) may contribute to the population of long-lived, quiescent mammary stem cells, which may be precursor cancer cells in the adult (**Figure 1C**). These findings imply the potential to simplify and deconvolute the study of tumors and their significant cellular and molecular heterogeneity by considering and focusing on subpopulations of cells having embryonic origins or molecular signatures.

This review provides a comprehensive summary of all known genes and signaling pathways that lead to aberrant embryonic mammary gland phenotypes in GEMMs as well as breast cancers and metastasis when dysregulated. Following this, recent studies that compare molecular signatures of the embryonic mammary glands and breast cancers are summarized. Evidence is provided to support the proposal that studying the development of the mammary glands aligns with providing an understanding of the mechanisms of cancer, with the intention to identify novel prognostic markers and therapeutic strategies against this prominent disease.

SIGNALING PATHWAYS IN EMBRYONIC MAMMARY GLAND DEVELOPMENT AND CANCER

WNT Signaling

WNT signaling regulates numerous developmental processes. The name "WNT" is a combination of wingless and

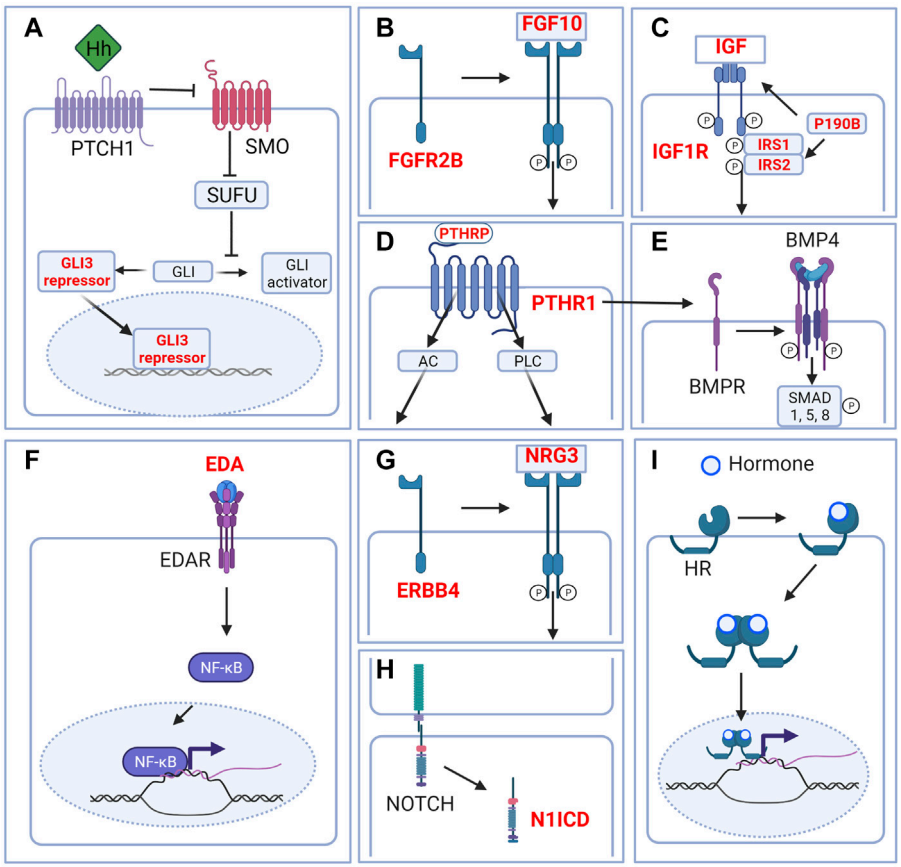


FIGURE 3 | Signaling pathways in the embryonic mammary gland. Cartoon depicting major signaling pathways that are critical during embryonic mammary gland development. **(A)** Hedgehog signaling. Although Hh ligands are expressed in the MRs, Hedgehog signaling is in the inactivated state. GLI is maintained in the repressor form to repress the transcription of Hh target genes. **(B)** FGF signaling. FGF10 binding to its main receptor, FGFR2B triggers receptor dimerization, phosphorylation and the activation of diverse downstream pathways. **(C)** IGF1R signaling. Ligand binding activates the receptor kinase, leading to receptor autophosphorylation, and tyrosine phosphorylation of multiple signaling adapter proteins including, the insulin receptor substrates (IRS1/2). **(D)** PTHRP signaling. PTHRP binding to the G-coupled receptor PTHR1 activates AC and PLC downstream signaling. **(E)** BMP signaling. Binding of ligand to the receptor complex stimulates BMPR autophosphorylation and phosphorylation of downstream substrates. BMP4 may interact with PTHRP signaling to facilitate epithelial-mesenchymal cross talk. **(F)** EDA-EDAR signaling. EDA binding to EDAR triggers downstream NF-kB signaling. **(G)** NRG3-ERBB4 signaling. NRG binding triggers receptor dimerization and activation of receptor tyrosine kinase domain and downstream signaling. **(H)** NOTCH signaling. Ligand binding triggers the cleavage of N1ICD which activates downstream signaling. **(I)** Hormone signaling. Binding of hormones such as estrogen or progesterone to their cognate hormone receptor (HR) promotes internalization of the hormone-receptor complex. Homo- or heterodimer formation ensues followed by translocation into the nucleus and binding to DNA response elements and transcription or repression of target genes. Only relevant components of each signaling pathway in embryonic mammary gland development are depicted. Genes highlighted in red denote genes that give rise to aberrant embryonic mammary gland phenotypes when deleted or overexpressed. P denotes phosphorylation events. Figure created with BioRender.

integration site-1 (Int1), of which the latter was identified as a genetic locus activated by the insertion of the MMTV that leads to the formation of mammary tumors (Nusse and Varmus, 1982). Presently, there are 19 known WNT ligands in mammals. Classical WNTs (WNT1, WNT3A, WNT8, and WNT8B) activate the canonical β-catenin pathway while non-classical WNTs (WNT4, WNT5A, and WNT11) activate the non-canonical WNT/calcium pathway.

The canonical WNT/β-catenin pathway primarily acts to regulate cytosolic β-catenin levels (**Figure 2**). Without WNT, β-catenin is targeted to the APC/AXIN destruction complex where it is phosphorylated by CK1 and GSK3β (Kimelman and Xu, 2006). Consequently, phosphorylated β-catenin is ubiquitinated by the E3 ubiquitin ligase β-TrCP and targeted for proteasomal degradation. In the absence of WNT, LEF/TCF transcription factors bind to WNT response elements, enabling the recruitment of co-repressors such as Groucho and histone deacetylases (HDACs) to promote chromatin compaction and inhibit target gene transcription. In the presence of WNT however, DVL recruitment by the frizzled receptor (FZD) results in the phosphorylation of co-receptors low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) and AXIN recruitment. This inhibits the AXIN-mediated phosphorylation and degradation of β-catenin, resulting in its accumulation and nuclear import. Nuclear β-catenin binds to LEF/TCF, replaces transcriptional repressors and recruits members of the switch/sucrose non-fermentable (SWI/SNF) family of transcriptional coactivators and other chromatin

remodellers. Transcriptional co-activators BCL9/LGL and pygopus (PYGO) aid in the transport of these proteins to the TCF/β-catenin complex. As the chromatin becomes less compacted and consequently more accessible, the transcription of WNT-target genes will proceed (Nusse and Clevers, 2017) (**Figure 2**).

WNT signaling plays critical roles in embryonic mammary gland development as inhibition and dysregulation of multiple pathway components results in aberrant phenotypes (**Table 1**, **Figure 2**). The absolute requirement for WNT signaling is demonstrated by the failure of MR induction caused by the expression of the secreted WNT inhibitor, Dickkopf-1 (*Dkk1*) under the ectoderm- and epithelium-specific *Krt5* promoter (Chu, Hens et al., 2004). In $Lef1^{-/-}$ embryos all MRs regress by E15.5, with MR2 and MR3 being the first to regress shortly after induction, highlighting the requirement of WNT signaling for MR maintenance (van Genderen et al., 1994; Boras-Granic et al., 2006). $Lrp5^{-/-}$ and $Lrp6^{-/-}$ embryos form hypoplastic MRs. Additionally, $Lrp6^{-/-}$ embryos display defects in branching morphogenesis (Lindvall et al., 2006; Lindvall et al., 2009). $Lrp4^{mdig/mdig}$ (*Lrp4* hypomorph) embryos display a delay in MR initiation as well as an aberrant number and distribution of mammary precursor cells leading to abnormal morphology, number and positioning of the MRs. In contrast to the previous WNT signaling-associated mutants, the $Lrp4^{mdig/mdig}$ mammary defects are associated with abnormally elevated WNT/β-catenin signaling. In support of this, $Lrp4^{mdig/mdig}$ mammary defects are abrogated by heterozygous or homozygous-null alleles of *Lrp5* and *Lrp6*, as well as the deletion of ectoderm and MR-specific β-catenin with the *Krt14-Cre* promoter ($Krt14\text{-}Cre;\beta\text{-}catenin^{flox/flox}$) (Ahn et al., 2013). *Lrp4* interacts with *Wise*/*Sostdc1* which modulates WNT signaling and inhibits bone morphogenetic protein (BMP) signaling (Itasaki et al., 2003; Laurikkala et al., 2003; Lintern et al., 2009). $Wise^{-/-}$ mice phenocopies a subset of the *Lrp4* mammary defects, including elevated WNT/β-catenin signaling whereas *Wise* overexpression reduces the number of mammary precursor cells. Finally, embryos null for *Pygo2* or lack ectoderm- and mammary epithelium-specific *Pygo2* ($Krt14\text{-}Cre;Pygo2^{flox/flox}$) show aberrant induction, sprouting and branching, most often in the thoracic MRs (Gu et al., 2009). Taken together, the levels of WNT/β-catenin signaling must be precisely regulated for proper MR development.

Despite there being a lack of mutations to pathway genes, an upregulation in WNT activity has been detected in most breast cancers and is linked to reduced overall survival (Zhan et al., 2017). High levels of β-catenin and its target gene, CCND1 were detected in 60% of breast cancers and correlates with poor prognosis (Lin et al., 2000). Decreased expression of the WNT inhibitory factor (WIF1) (Ai et al., 2006) and an elevated expression of WNT ligands are commonly observed in breast cancer (Xu et al., 2020). Aberrant epigenetic changes, including methylation of the APC gene promoter which could dysregulate WNT signaling has also been detected in inflammatory breast cancer (Van der Auwera et al., 2008). Importantly, WNT hyperactivity may result due to other cancer-associated mutations and aberrant activation of cancer-associated signaling pathways. For example, stabilization of β-catenin by WNT-independent pathways, such as PIN1, P53, PTEN/AKT, and NF-κB pathways, plays a significant role in breast cancer and malignant progression (Incassati et al., 2010). Intriguingly, the embryonic transcription cofactor limb-bud and heart (LBH), a direct target of WNT signalling in epithelial development, is overexpressed in the basal subtype of breast cancer (Rieger et al., 2010); this is suggestive of a WNT-mediated reversion to an embryonic-like state during tumorigenesis.

WNT signaling activation is implicated in metastasis. Cytokine signaling from the local bone microenvironment activates NF-κB and cAMP response element-binding protein (CREB) signaling in breast cancer cells, which in turn, initiates an autocrine WNT signaling loop. This leads to CSC colony formation in the bone marrow (Eyre et al., 2019). Importantly, inhibition of WNT signaling by recombinant human DKK1 or anti-FZD is sufficient to prevent metastatic colonisation in the bone. Other therapeutic strategies to inhibit WNT signaling include the use of small molecule inhibitors against the FZD receptor, destruction complex, nuclear β-catenin, and WNT ligand modifying enzymes such as Porcupine and Tankyrase (Wen et al., 2020).

Hedgehog Signaling

The Hedgehog (HH) pathway is activated by HH ligand binding to patched (PTCH), a transmembrane transporter-like protein located at the cilium. Upon HH binding, the suppression of PTCH on the seven-span transmembrane protein smoothened (SMO) is released, leading to the release of SMO-mediated inhibition on the suppressor of fused (SUFU) (Wang et al., 2007). As a result, phosphorylation and proteolytic processing of the GLI family of zinc-finger transcription factors are inhibited and full-length proteins that function as transcriptional activators accumulate (Kise et al., 2009; Humke et al., 2010). GLI1, GLI2, and GLI3 mediate HH signaling in vertebrates; whereas GLI1 is exclusively a transcriptional activator, GLI2 and GLI3 could either activate or repress transcription (**Figure 3A**).

HH ligands namely Sonic hedgehog (*Shh*) and Indian hedgehog (*Ihh*) are expressed in the MRs at E12.5. Despite this, $Shh^{-/-}$ embryos induce all MRs and show normal branching morphogenesis at E16.5 (Michno et al., 2003). Similarly, transplantation of $Shh^{-/-}$ and $Ihh^{-/-}$ MRs into wildtype cleared fat pads results in normal branching morphogenesis. Taken together, these observations indicate that epithelial *Shh* and *Ihh* are dispensable for MR induction and branching morphogenesis (Gallego et al., 2002).

Gli1 is a reliable marker of HH signaling activation as *Gli1* is a direct transcriptional target of HH signaling and its expression is strictly dependent on HH signaling pathway activation transduced by either *Gli2* or *Gli3* transcriptional activators (Dai et al., 1999). While *Gli1* expression is not detected in the somites underlying MR3 at E11.5, *Gli2* and more prominently, *Gli3*, are expressed. *Gli1* expression is absent in the MRs from E11.5 to E14.5. This indicates that HH signaling is inactive in the somites and MRs in these stages (Hatsell and Cowin, 2006). The upregulation of *Gli1* and, to a lesser extent, *Gli2* in $Gli3^{Xt\text{-}J/Xt\text{-}J}$ (*Gli3* null) MRs indicates that *Gli1* expression is suppressed by *Gli3* repressor during normal MR development. Furthermore, the

loss of *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ MR3 and MR5 is phenocopied by the expression of *Gli1* under the *Gli2* promoter and *Gli3* heterozygosity (Gli2$^{1nki/1nki}$; Gli3$^{Xt/+}$). Altogether, this shows that HH signaling must be inactivated for MR3 and MR5 induction (Hatsell and Cowin, 2006; Lee et al., 2013). Moreover, aberrant activation of HH signaling in *Gli3*$^{Xt\text{-}J/Xt\text{-}J}$ embryos results in hypoplasia as well as defective bud and branching morphogenesis in MR2, MR4, and to a lesser extent, MR1 (Lee et al., 2011). *Gli1*$^{lzki/lzki}$ (*Gli1* null) and *Gli2*$^{lzki/lzki}$ (*Gli2* null) embryos induce all MRs normally (Hatsell and Cowin, 2006).

In contrast to embryonic mammary gland development, accumulating evidence suggests that HH signaling is activated during tumorigenesis. Conditional over-expression of GLI1 with the MMTV promoter expands mammary progenitor cells, upregulates genes involved in proliferation, cell survival, EMT and metastasis and results in tumors that display the malignant basal or hybrid basal and luminal epithelial phenotypes (Fiaschi et al., 2009). Noteworthy, HH signaling components and other oncogenic pathways integrate to synergistically promote breast tumorigenesis. Specifically, GLIs may be modulated by non-HH signaling pathways through the integration of signals from TGFβ (Javelaud et al., 2011) and NF-κB (Colavito et al., 2014) signaling pathways. Similarly, although *Gli3* does not have a direct role in breast cancer, it has been found to cooperate with other genes such as androgen receptor (AR) (Lin et al., 2022), estrogen receptor (ER) (Massah et al., 2021) and *Eph10A* (Peng and Zhang, 2021) to promote tumorigenesis and invasive phenotypes. In silico analyses of gene expression profile datasets identified GLI3 as a putative interacting partner of TBX3, an important regulator in embryonic mammary gland development commonly overexpressed in breast cancer (see also section on TBX below). Further sequence-based and chromatin immunoprecipitation analyses show that *Gli3* is a direct transcriptional target of TBX3 (Mosca et al., 2009).

The HH signaling pathway is also activated in the CSC-enriched CD44^{+}CD24$^{-/low}$ population and side population of the MCF7 breast cancer cell line (Tanaka et al., 2009). The interaction of PTCH1 with the membrane glycoprotein, TSPAN8, leads to enhanced SHH signaling, increased tumor formation in mice and resistance to chemotherapeutic agents (Zhu et al., 2019). These observations suggest a link between the deregulation of HH signaling and the acquisition of cancer stemness and therapeutic resistance. Finally, in metastasis, paracrine signaling and activation of the HH pathway in stromal cells *via* tumor cell HH ligand overexpression increases invasiveness and metastasis in breast cancer (O'Toole et al., 2011).

The HH pathway has been extensively explored as therapeutic targets for breast cancer (Bhateja et al., 2019). Treatment of cyclopamine, a naturally-occurring steroidal alkaloid that inhibits the HH pathway by binding to SMO, suppresses *Gli1* expression and the growth of HH pathway-activated breast carcinoma cells (Kubo et al., 2004). GLI inhibitors have also been extensively developed for clinical trials (Bhateja et al., 2019; Riobo-Del Galdo et al., 2019).

Fibroblast Growth Factor Signaling

The fibroblast growth factor (FGF) family is composed of at least 22 members and 4 FGF receptors (FGFRs) that are involved in various aspects of vertebrate development (Pownall and Isaacs, 2010). Upon ligand binding, FGFRs dimerize and become phosphorylated in the intracellular tyrosine kinase domains, which, in turn, leads to the activation of various downstream proteins; e.g., FGFR substrate 2 (FRS2), phospholipase Cγ (PLCγ), among others. FGF signals are typically transduced by the RAS/MAPK, PI3K/AKT, or PLCγ downstream cascades which regulate a myriad of cellular processes like cell growth. Given its wide-ranging effects, FGFR signaling is tightly regulated, exemplified by its negative regulation by MAPK phosphatase 3, Sprouty proteins, and similar expression to FGF (SeF) family members (Santolla and Maggiolini, 2020) (**Figure 3B**).

FGF10 and its main receptor, FGFR2B are involved in epithelial-mesenchymal interactions, as suggested by their complementary expression domains in the MRs. At E10.5, *Fgf10* is expressed in the ventral region of the thoracic somites (Mailleux et al., 2002; Veltmaat et al., 2006). From E11 to E12, *Fgfr2b* is expressed in the mammary epithelium (Spencer-Dene et al., 2001; Mailleux et al., 2002). Strikingly, *Fgf10*$^{-/-}$ embryos only form MR4 (Mailleux et al., 2002; Veltmaat et al., 2006). Even so, development of MR4 proceeds aberrantly as at E18.5, *Fgf10*$^{-/-}$ MR4 consists only of a sprout connected to the nipple, demonstrating the involvement of *Fgf10* in branching morphogenesis in addition to MR induction. The requirement of FGF signaling in branching morphogenesis is consistent with the finding that at E18.5, all 4 FGFRs (most prominently FGFR2 and FGFR3) are expressed in the MRs (Spike et al., 2012). Like *Fgf10*$^{-/-}$ embryos, *Fgfr2b*$^{-/-}$ embryos only induce MR4 (Veltmaat et al., 2006), albeit transiently as it regresses by E13 due to mammary epithelial apoptosis. Intriguingly, high levels of FGF10 are required for MR3 induction as *Fgf10*$^{mlcv/-}$ (*Fgf10* hypomorph) embryos lack MR3 (Veltmaat et al., 2006). This suggests MR-specific requirement for FGF signaling activation.

Fgf7, another ligand of *Fgfr2b*, is expressed at E12.5 in the mesenchyme, before the formation of the mammary mesenchyme. This suggests that FGF7 may act redundantly with FGF10 to activate FGFR2B for MR4 maintenance. By E15.5, *Fgf7* expression decreases but expands into the adjacent fat pad precursor (Mailleux et al., 2002). Other ligands of FGFR2B namely *Fgf1* and *Fgf3* are not expressed in the MRs.

FGF signaling misregulation is common across all breast cancer subtypes (Santolla and Maggiolini, 2020). FGFR1 amplification is the most frequent genomic alteration whereas FGFR2-4 amplification is relatively less common in breast cancer (Reis-Filho 2006). Interestingly, FGFR1 amplification may be breast cancer subtype-specific: in particular, the FGFR1 locus (8q12) is amplified in nearly 15% of hormone receptor-positive breast cancers but only in 5% of triple-negative breast cancers (TNBC). Aberrant activation of FGF/FGFR signaling caused by FGF overexpression or FGFR1 amplification and overexpression in ER^{+} breast cancer cells is associated with estrogen-independent cell proliferation, metastasis and reduced distant metastasis-free survival (McLeskey et al., 1998; Turner and Grose, 2010). These

studies, and many others, strongly suggest the involvement of FGF signaling in malignant progression. As such, various therapeutic approaches have been developed to inhibit the pathway including small molecule inhibitors and monoclonal antibodies (mAbs) that block FGFRs or entrap FGFs (Santolla and Maggiolini, 2020).

P190b RhoGTPase Activating Protein, Insulin Receptor Substrate, and Insulin-Like Growth Factor 1 Receptor Signaling

P190b is a member of the RhoGAP family which are negative regulators of RhoGTPase activity (Burbelo et al., 1995). At E12.5, *p190b* is expressed in the mammary epithelium while at E14.5 *p190b* expands its expression domain, but at a lower level, to the adjacent mesenchyme (Heckman et al., 2007). *P190b*$^{-/-}$ embryos develop hypoplastic MRs with disorganized mammary mesenchyme that lack AR expression (Heckman et al., 2007). Thus, *p190b* is required for MR growth, mammary mesenchyme specification, maturation and potentially sexual dimorphism. At E14.5, *p190b*$^{-/-}$ MRs show a decrease in the expression of adaptor proteins, insulin receptor substrate 1 (IRS1) and IRS2 and the insulin-like growth factor 1 receptor (IGF1R) signaling marker, phosphorylated Akt. IRS1 and IRS2 are expressed in the mammary epithelium and mesenchyme at E14.5. Similar to *p190b*$^{-/-}$ embryos, *Irs1*$^{-/-}$;*Irs2*$^{-/-}$ compound mutants develop hypoplastic MRs and lack mammary mesenchyme differentiation at E14.5 (Heckman et al., 2007). Finally, and consistent with the previous mutants, *Igf1r*$^{-/-}$ embryos develop hypoplastic MRs at E14.5. Taken together, P190B, IRS1, IRS2, and IGF1R form a signaling network that regulates various aspects of embryonic mammary development (**Figure 3C**).

Although RhoGTPases are commonly overexpressed and hyperactivated in breast cancers (Tang et al., 2008), paradoxically, *P190b* has been implicated as an oncogene in breast carcinogenesis. *P190b* haploinsufficiency inhibits MMTV-Neu tumor formation, progression, angiogenesis, and metastasis (Heckman-Stoddard et al., 2009). Consistently, specific overexpression of p190b in the mammary epithelium, also in MMTV-Neu mice, led to enhanced tumorigenesis and metastasis mediated by downstream Rac1-dependent reactive oxygen species (ROS) production (McHenry et al., 2010).

Despite their homology, IRS1 and IRS2, have distinct functions in regulating breast cancer progression (Gibson et al., 2007). Specifically, IRS2 is a positive regulator of metastasis (Nagle et al., 2004), whereas IRS1 is a suppressor of metastasis in the MMTV-PyMT mouse model (Ma et al., 2006). More recently, IRS1 has also been implicated in progesterone receptor (PR)-driven stemness and endocrine resistance in ER^{+} breast cancer (Dwyer et al., 2021). The IGF1R-IRS1/2 signaling axes may be important in breast cancers as at least 50% of breast tumors have activated IGF1R (Ekyalongo and Yee, 2017).

Several strategies have been developed to inhibit IGF1R signaling including the use of mAbs, small-molecule tyrosine kinase inhibitors of IGF1R and insulin receptor, and ligand neutralization. However, clinical trials show no appreciable benefit of these approaches thus far (Ekyalongo and Yee, 2017). A more promising approach uses the small-molecule tyrphostin, NT157 to target IRS; this method downregulates IRS protein expression and sensitizes ERα^{+} breast cancer cells to the mammalian target of rapamycin (mTOR) inhibitor, rapamycin. Moreover, NT157 inhibits the growth of tamoxifen-resistant ERα^{+} breast cancer cells (Yang et al., 2018).

Parathyroid Hormone-Related Protein Signaling

Parathyroid hormone-related protein (PTHRP) derives its name from and shares structural and functional similarities with parathyroid hormone (PTH). PTHrP and PTH bind to and signal through the Type 1 PTH/PTHRP receptor (PTHR1) which is a G-protein coupled receptor. Activation of PTH1R causes an acute increase in intracellular signaling molecules including the two classical G protein signaling cascades initiated by adenylate cyclase (AC) and phospholipase C (PLC). This leads to a variety of responses including the transcription of target genes and the regulation of intracellular calcium levels (Hiremath and Wysolmerski, 2013) (**Figure 3D**).

The complementary expression pattern of *Pthrp* and *Pthr* in the embryonic mammary gland is indicative of their roles in mediating epithelial-mesenchymal interactions. *Pthrp* is expressed in the mammary epithelium from the placodal stage through birth, whereas *Pthr1* is expressed in the mammary and dermal mesenchyme (Wysolmerski et al., 1998). Disruption to either gene results in the induction of normal MRs, however nipple formation and subsequent development are impeded (Wysolmerski et al., 1998). *Pthrp* has non-cell autonomous roles as it is necessary for the differentiation and maturation of the mammary mesenchyme. In *Pthrp*$^{-/-}$ embryos, mesenchymal cells lack AR and tenascin C expression, although they continue to condense around the epithelial bud (Wysolmerski et al., 1998; Dunbar et al., 1999; Hiremath et al., 2012). The lack of AR expression causes the MRs of *Pthrp*$^{-/-}$ and *Pthr1*$^{-/-}$ male embryos to resist androgen-mediated destruction that is essential for sexual dimorphism (Dunbar et al., 1999). Conversely, overexpression of *Pthrp* under the control of the ectoderm- and MR-specific *Krt14* promoter results in aberrant mammary mesenchyme differentiation and supernumerary nipple formation in the ventral epidermis (Foley et al., 2001). Taken together, PTHRP-PTHR1 signaling is critical for epithelial-mesenchymal cross-talks, sexual dimorphism, mesenchymal maturation, and branching morphogenesis.

Pthrp upregulates bone morphogenetic protein receptor 1a (*Bmpr1a*) expression in the mammary mesenchyme to enable BMP4-mediated signaling. Ductal outgrowth regulated by PTHrP may be mediated in part by BMP4 as its supplementation can rescue ductal outgrowth defects in *Pthrp*$^{-/-}$ MRs. This points to critical roles of BMP signaling in the mesenchyme and in the development of the MRs after E12.5 (Hens et al., 2007) (see section on BMP signaling). The specification of the mammary mesenchyme may also implicate the homeobox transcription factor *Msx2* (Hens et al., 2007) (see also section on HOX transcription factors) and canonical WNT signaling in the

mesenchyme, as its expression and activity, respectively, are dependent on *Pthrp* (Hiremath et al., 2012).

PTHRP and PTHR1 are often overexpressed in breast cancer and notably, PTHR1 is commonly overexpressed in breast cancer stroma (Henderson et al., 2006). Genome-wide association studies have implicated both the parathyroid hormone-like hormone (PTHLH) and the PTHR1 loci as breast cancer susceptibility genes (Garcia-Closas et al., 2013; Michailidou et al., 2013). In line with this, loss of *Pthrp* in the mammary epithelium of the MMTV-PyMT mice delays tumor initiation, progression, and reduces metastasis. Besides regulating genes involved in cell proliferation, angiogenesis, and apoptosis, PTHRP also affects the expression of adhesion factor CXCR4, which may be crucial for metastatic dissemination (Li et al., 2011).

PTHRP is important for metastatic colonisation in various distal sites, in particular the bone through the upregulation of osteoblastic receptor activator of NF-κB ligand (RANKL) which drives bone destruction, and downregulation of osteoprotegerin (OPG) expression (Guise et al., 2002). Consistent with this, a neutralising mAb against PTHRP diminished tumor growth and lytic bone lesions in MDA-MB-231 human breast cancer mice xenografts (Guise et al., 1996). PTHRP-specific neutralizing antibodies have also been shown to reduce lung metastases in MDA-MB-435 mice xenografts (Li et al., 2011), suggesting the broad utility of such antibodies and the feasibility of PTHRP as a target for metastasis.

Bone Morphogenetic Protein Signaling

Bone morphogenetic proteins (BMPs) constitute a large family of secreted growth factors from the transforming growth factor beta (TGFβ) family of ligands that are involved in many aspects of development. The binding of BMPs to their cognate receptors results in the phosphorylation of the "Small", receptor-regulated "Mothers Against Decapentaplegic" homolog (SMAD) family members, notably SMAD1, SMAD5, and SMAD 8. Phosphorylated SMAD 1/5/8 forms a complex with SMAD4 that translocates into the nucleus to regulate the expression of target genes (Chen et al., 2004; Wang et al., 2014) (**Figure 3E**).

Bmp2 is expressed in the mammary epithelium at E13.5 (Phippard et al., 1996), although its function remains to be elucidated as $Bmp2^{-/-}$ mutants are not viable before the onset of mammogenesis. From E11.5 to E14.5 *Bmp4* is expressed predominantly in the mesenchyme and at lower levels in the mammary epithelium (Phippard et al., 1996; Hens et al., 2007). *Bmp4* is involved in various processes in the embryonic mammary gland including the positioning of the mammary line along the dorso-ventral axis, along with *Tbx3*, at E11.5 (Cho et al., 2006), epithelial-mesenchymal maturation and ductal outgrowth in conjunction with its receptor, *Bmpr1a* (Hens et al., 2007). *Bmpr1a* expression in the mesenchyme is regulated by PTHRP (see section on PTHRP signaling).

Aberrant expression of BMPs and misregulation of BMP signaling has been associated with breast cancer; however, their roles and effects in tumorigenesis can be context-dependent and ligand-specific. Several BMPs, including BMP2, BMP6, BMP9, BMP10, BMP15, and GDF9a inhibit the proliferation of breast cancer cells, whereas BMP4 and BMP7 can either promote or inhibit proliferation in different contexts (Alarmo and Kallioniemi, 2010; Zabkiewicz et al., 2017).

Besides proliferation, BMPs can also regulate cancer cell stemness. Recombinant BMP2 induces EMT and stemness of breast cancer cells *via* the Rb and CD44 signaling pathways, which leads to metastasis (Huang et al., 2017). In contrast, BMP4 may act as an autocrine mediator to activate SMAD7 and block metastasis in animal models of breast cancer. Restored BMP4 expression or therapeutically administered BMP4 protein sensitizes cancer cells to anoikis, reduces the number of circulating tumor cells and the extent of metastasis, thereby resulting in increased survival (Eckhardt et al., 2020).

Various approaches have been developed to modulate BMP signaling, including the use of BMPR kinase inhibitors and other soluble decoy receptors which can prevent the interaction of BMPs in the extracellular space with membrane-embedded receptors. Downregulation of SMAD signaling *via* the silencing of the E3 ubiquitin ligase, Smurf1 also attenuates BMP signaling (Lowery and Rosen, 2018).

Ectodysplasin Signaling

Ectodysplasin (*Eda*) is a member of the tumor necrosis factor (TNF) ligand superfamily, and functions with its receptor, *Edar*, to regulate the development of a variety of ectodermal appendages (**Figure 3F**). Upon EDA binding, EDAR recruits the adaptor protein EDARADD and *via* TRAF6 activates the IKK complex. This leads to the phosphorylation of I-κB, translocation of NF-κB into the nucleus and target gene transcription (Mikkola and Thesleff, 2003) (**Figure 3F**).

Edar is expressed in the mammary epithelium at E12 (Pispa et al., 2003) whereas *Eda* is expressed in the mesenchyme before and during branching morphogenesis from E15.5 to E17.5 (Voutilainen et al., 2012). Although $Eda^{-/-}$ (Tabby) mutants have normal numbers of mammary glands, the glands have smaller ductal trees with fewer branches (Mustonen et al., 2003). Conversely, overexpression of *Eda* using the *Krt14* promoter induces supernumerary MRs which develop into mature glands in the adult, between MR3 and MR4 (Mustonen et al., 2003; Mustonen et al., 2004). *Krt14-Eda* MRs display precocious branching morphogenesis and ectopically activate NF-κB. EDA signaling is likely mediated by NF-κB as inhibition of NF-κB, concurrent with *Eda* overexpression result in smaller ductal trees with fewer branches at E18 (Voutilainen et al., 2012). Important regulators of embryonic mammary gland development namely *PTHrP*, *Wnt10a*, *Wnt10b* and other genes such as the epidermal growth factor (EGF) family ligands, *Areg* and *Epgn*, have been identified as potential transcriptional targets of EDA/NF-κB signaling during ductal development, suggesting the integration of these pathways for MR development.

Krt14-Eda mice do not show palpable tumors, however, elevated EDAR signaling in $Edar^{Tg951}$ (*Edar* copy-number amplification) transgenic mice results in a high incidence of mammary tumors in breeding female mice. These tumors may bear important, clinical-relevant characteristics as they resemble *EDAR*-high human tumors which lack ER expression but have

elevated β-catenin transcriptional activity and extensive squamous metaplasia (Williams et al., 2022).

Neuregulin 3

The neuregulin (Nrg) family consists of four genes, *Nrg1*, *Nrg2*, *Nrg3*, and *Nrg4*, which is characterized by a conserved domain related to the EGF family of ligands. *Nrg3* is a ligand for the receptor tyrosine kinase erythroblastic leukaemia viral oncogene homolog 4 (ERBB4) that belongs to the ErbB receptor tyrosine kinase family (Zhang et al., 1997). Ligand binding causes the receptor to dimerize and activate intracellular tyrosine kinase domain, leading to the activation of downstream signaling cascades such as the PI3K/AKT and MAPK pathways to regulate various processes (Stern, 2000; Hayes and Gullick, 2008) (**Figure 3G**).

In embryonic mammary gland development, *Nrg3* is likely involved in early inductive events as it is expressed in the dermal mesenchyme adjacent to the presumptive mammary region and the presumptive mammary region itself at E11 (Howard et al., 2005). Subsequently, *Nrg3* is expressed in the mammary epithelium. Like *Nrg3*, *Erbb4* is expressed in the dermal mesenchyme underlying the presumptive MR3 and MR4 at E11.5. Subsequently, *Erbb4* is expressed in the mammary epithelium and ectoderm at E12.5 and E13 (Howard et al., 2005).

Scaramanga ($Nrg3^{ska}$, *Nrg3* hypomorph) embryos often fail to induce MR3 but induce supernumerary MRs adjacent to the site of MR4, suggesting the anatomical region-specific roles of *Nrg3* in mammogenesis. Application of recombinant NRG3 or *Nrg3* overexpression using the *Krt14* promoter induces MRs along the mammary line (Howard et al., 2005; Panchal et al., 2007). $Nrg3^{ska}$ MR3 display defects in mammary mesenchyme specification characterized by the downregulation of *Lef1*, *ER*, *AR*, and *Pth1r* expressions at E12.5 (Kogata et al., 2014).

In human breast cancer cell lines, NRG3 activates ectopically-expressed ERBB receptors (ERBB1-4). Whereas NRG3 is potentially overexpressed in breast cancer, paradoxically, recombinant NRG3 diminished the growth of human breast cancer cells *in vitro*. These results indicate potential dose-dependent effects of NRG3 (Hijazi et al., 1998). On the other hand, ERBB family receptor tyrosine kinases are commonly overexpressed in breast cancers, in particular, *ErbB2* or HER2/neu amplification constitute a major breast cancer subtype found in 15–30% of breast cancers while *ErbB4* overexpression is less common. Intriguingly, *ErbB4* has context-dependent tumor suppressive and oncogenic roles (Sundvall et al., 2008). Therapeutics targeting ERBB receptors in breast cancer include the humanized anti-ErbB2 antibody trastuzumab (Herceptin) and the tyrosine kinase inhibitor, lapatinib which are efficacious and widely used in the clinic.

NOTCH Signaling

The NOTCH pathway mediates juxtacrine cellular signaling where transmembrane ligands on one cell activate transmembrane receptors on a juxtaposed cell (Hori et al., 2013; Siebel and Lendahl, 2017). Four receptors (NOTCH1–4) and five ligands—Delta-like ligand 1, 3, 4 (DLL1, 3, 4), Jagged 1 and 2 (JAG1, 2)—have been described in mammals (Hori et al., 2013) (**Figure 3H**).

NOTCH signaling is activated upon the binding a NOTCH ligand to its receptor, which triggers receptor cleavage by a member of the disintegrin and metalloprotease domain family (ADAM17 or ADAM10) and a presenilin-dependent γ-secretase complex. The cleaved intracellular domain of the NOTCH receptor (NICD) translocates into the nucleus where it forms a complex with the DNA-binding protein, CSL, and other transcriptional co-activators to drive Notch-target genes expression (Kopan and Ilagan, 2009; Hori et al., 2013).

Several *in vivo* lineage tracing studies demonstrate that at the population level, embryonic mammary gland cells are multipotent, bearing the capacity to give rise to basal and luminal cell lineages in the postnatal mammary gland (Van Keymeulen et al., 2011; Wuidart et al., 2016). However, NOTCH1 activation *via* the transgenic overexpression of NOTCH1 intracellular domain (N1ICD)—a ligand-independent active form of the NOTCH1 receptor—imposes a luminal $ERα^-$ cell fate onto E13.5 cells (Lilja et al., 2018). This suggests that NOTCH signaling must be inactivated to maintain the multipotency of the embryonic mammary cells (see section on cellular lineages and stem cell potency).

NOTCH signaling is frequently deregulated in different breast cancer subtypes and is associated with the acquisition of therapeutic resistance (Lamy et al., 2017; Krishna et al., 2019; Nandi and Chakrabarti, 2020). Overexpression of the NOTCH1 intracellular domain with the MMTV promoter [MMTV-Notch1 (intra)] impairs mammary gland development and induces mammary tumors, suggesting the oncogenic role of NOTCH1 (Hu et al., 2006). Other studies also show NOTCH1 activation and its association with metastatic breast cancer cells (Mohammadi-Yeganeh et al., 2015). Interestingly, accumulating evidence points to the involvement of juxtacrine NOTCH signaling between tumor cells and cells that constitute the tumor microenvironment such as immune cells, cancer associated fibroblasts and endothelial cells to promote malignant progression (Meurette and Mehlen, 2018).

Gamma-secretase inhibitors (GSIs) are pan-NOTCH inhibitors that are the first and most extensively explored small molecules targeting the NOTCH pathway. GSIs reduce the levels of NICDs and several other substrate proteins, thereby inhibiting downstream signaling. Other promising approaches include anti-Notch mAbs which target the receptor-ligand binding domain or the negative regulatory region (NRR) of the NOTCH receptor, and, in turn, block intracellular NOTCH cleavage by γ-secretases and signal transduction (Lamy et al., 2017).

Homeobox Transcription Factors

Homeotic (*Hox*) genes encode for the prototypic homeobox transcription factors, which are known to be master regulators of developmental programs (Carroll, 1995). The role of *Hox* genes in regional specification is reflected in their sequential, partially overlapping expression domains along the antero-posterior body axis. This is also reflected in the relative positions of the *Hox* genes on the chromosome (Morgan, 1997).

Several homeobox factors are involved in MR development: *Hoxc6*$^{-/-}$ mouse embryos form hypoplastic thoracic MRs at E12.5 but their inguinal MRs are unaffected (Garcia-Gasca and Spyropoulos, 2000). The position-specific phenotype is consistent with the expression of *Hoxc6* in the anterior somites underlying the thoracic MRs, and its absence in the posterior somites underlying inguinal MRs. *Hoxb9* and *Hoxd9* are expressed in the mammary mesenchyme at E12.5 (Chen and Capecchi, 1999), although their function, if any, in embryonic mammary gland development is unknown. Supernumerary mammary gland formation has been attributed to the ectopic expression of *Hox* genes (Schmidt, 1998). Taken together, *Hox* genes are important for MR-specific induction and growth.

The *Msx* genes belong to a small family of three homeobox-containing transcription factors related to the muscle segment homeobox gene, *msh*, in Drosophila (Davidson, 1995). *Msx1* and *Msx2* are expressed in the mammary epithelium at E13.5 (Phippard et al., 1996) whereas at E14.5, *Msx2* is expressed in the mesenchyme (Satokata et al., 2000). *Msx1*$^{-/-}$ MRs develop normally (Phippard et al., 1996) while in *Msx2*$^{-/-}$, MR4 development arrests at the sprout stage by E16.5. *Msx1*$^{-/-}$; *Msx2*$^{-/-}$ compound mutants form a hypoplastic, protruded MR4 coincident with defective mammary mesenchyme at E15.5. These aberrant phenotypes are linked to the loss of *Lef1* expression in the epithelium and mesenchyme (Satokata et al., 2000). Thus, *Msx1* and *Msx2* play non-redundant roles in MR development. Lastly, the paired-box homeobox gene 3 (*Pax3*) is expressed in the somites at E11.5 (Veltmaat et al., 2006). *Pax3*$^{ILZ/ILZ}$ (*Pax3* null) mouse embryos do not form hypaxial buds of the somites and show delayed induction of MR3; this finding highlights the role of the somites for MR3 induction (Veltmaat et al., 2006).

Hox transcription factors play multiple roles in breast cancer including cell cycle control, apoptosis, angiogenesis and cell-cell communication (Lewis, 2000; Briegel, 2006). HOXB7 has been reported as an oncogene associated with the upregulation of bFGF expression (Care et al., 1998) and EMT induction (Wu et al., 2006). Bisphenol-A, a known endocrine-disrupting compound that increases the risk of breast cancer, induces the expression of estrogen-regulated *Hoxc6* (Hussain et al., 2015) and *Hoxb9* (Deb et al., 2016). Comparatively, other *Hox* family members like HOXA9 may exhibit tumor suppressive roles by upregulating the expression of BRCA1 in breast cancer cells. Moreover, HOXA9 downregulation is associated with elevated tumor invasion, metastasis, and patient mortality (Gilbert et al., 2010). *Hox* genes may engage epigenetic regulators to regulate tumorigenesis and metastasis (Sun et al., 2013).

One potential strategy to inhibit HOX function is *via* the use of HXR9 peptides which prevents HOX binding to PBX, a transcription co-activator common to many HOX proteins. HXR9 causes apoptosis in multiple breast cancer cell lines (Morgan et al., 2012). Overcoming the functional redundancies among the different HOX family members is one of the main challenges for HOX-based therapeutic strategies (Feng et al., 2021).

T-Box Transcription Factors

The T-box family (TBX) are transcriptional activators or repressors that are defined by a highly conserved T-box DNA binding domain (Wilson and Conlon, 2002). *Tbx2* is expressed in the mammary mesenchyme at E11.5 while *Tbx3* is expressed in the mammary epithelium between E11.5 and E12.5 (Chapman et al., 1996; Eblaghie et al., 2004). *Tbx3* expression is restricted to the dorsal domain by ventral *Bmp4* expression, which also determines the position of the mammary line (Cho et al., 2006). *Tbx3*$^{tm1Pa/+}$ (*Tbx3* heterozygous) mice form mammary placodes but maintenance of a subset of thoracic buds, nipple formation and ductal branching are impaired (Jerome-Majewska et al., 2005). Expectedly, *Tbx3*$^{tm1Pa/tm1Pa}$ (*Tbx3* null) embryos have a more severe phenotype, failing to induce most MRs altogether (Davenport et al., 2003). *Tbx2*$^{tm1Pa/+}$ (*Tbx2* heterozygous) embryos form MRs normally whereas *Tbx2*$^{tm1Pa/tm1Pa}$ (*Tbx2* null) embryos show inductive defects for MR2 and MR5.

Although induction is largely not affected in *Tbx2*$^{+/-}$;*Tbx3*$^{+/-}$ compound mutants, some thoracic MRs regress by E18.5. For MRs that do progress, nipple formation and branching morphogenesis are frequently affected (Jerome-Majewska et al., 2005). In sum, *Tbx* genes play important roles in the induction, maintenance, nipple formation and branching morphogenesis of the MRs.

Both TBX2 and TBX3 are deregulated in breast cancers. Interestingly, TBX2 is found in a region of amplification on chromosome 17q23, which is common to about 20% of human breast cancers (Sinclair et al., 2003). TBX2 may be involved in malignant progression as its overexpression correlates with advanced tumor stages and with aggressive, hereditary BRCA1/2 breast cancers. Mechanistically, the deregulation of TBX2 or TBX3 may result in the bypass of P53-mediated senescence, growth arrest and apoptosis in breast cancers. TBX2 and TBX3 suppress *Cdnk2a/p19Arf* (*p14Arf* in human) transcription, which induces cell cycle arrest at the G1 and G2 phase by interfering with MDM2, a negative regulator of P53 (Briegel, 2006). The repression of *p14Arf* by TBX3 overexpression may be mediated by HDACs (Yarosh et al., 2008). Interestingly, estrogen signaling expands breast CSCs in MCF7 breast cancer cells through a paracrine FGF/FGFR/TBX3 signaling pathway, suggesting a role for *Tbx3* in promoting stemness (Fillmore et al., 2010). On the other hand, *Tbx2* has been shown to repress the expression of *p21WAF1/CIP1*, a P53 target necessary for P53-mediated growth arrest (Prince et al., 2004). TBX2 overexpression directly represses E-cadherin transcription and promotes EMT (Wang et al., 2012). Importantly, TBX2 and TBX3 may additionally play non-redundant roles in breast cancers. For example, TBX2, but not TBX3, is associated with increased metastatic potential of breast tumors through its regulation of adhesion molecules like cadherins and integrins (Rowley et al., 2004). Therapeutic strategies for TBX proteins, currently unavailable, could be directed towards their unique small repression domains (Chang et al., 2016).

GATA3

GATA3 belongs to a family of zinc finger transcription factors that bind to a consensus DNA sequence (A/T)GATA (A/G) in gene promoter regions to directly activate or repress target gene expression (Du et al., 2015).

GATA3 is expressed in the mammary epithelium and ectoderm at E12.5 (Asselin-Labat et al., 2007). Conditional

deletion of *Gata3* in these compartments under the *Krt14* promoter (*Krt14–Cre*;*Gata-3*$^{flox/flox}$) results in the lack of induction and hypoplasia of a variable subset of MRs, as assessed by *Lef1* expression at E11.75 (Asselin-Labat et al., 2007).

GATA3 is one of the most frequently mutated genes in breast cancer and has context-dependent tumor suppressive or oncogenic roles. GATA3 heterozygosity in MMTV-PyMT mice expands the CD14^{+} c-kit$^{-/lo}$ and c-kit^{+} luminal progenitor cell population and promotes tumorigenesis; contrastingly, overexpression of GATA3 in the same mouse model promotes cellular differentiation, reduces angiogenesis and inhibits tumorigenesis (Asselin-Labat et al., 2011). In MCF7 human breast cancer cells, GATA3, through its transcription regulation of CCND1 and in association with PARP1 promotes cell proliferation and tumorigenesis by facilitating the G_1 to S phase transition in the cell cycle. *In vivo* studies further show that GATA3 knockdown dramatically reduces tumor volume (Shan et al., 2014).

In the context of metastasis, GATA3 overexpression in LM2-4175 breast cancer cell line, an aggressive derivative of MDA-MB-231, inhibits cancer cell expansion in the lung. This is linked to GATA3-mediated downregulation of ID1/-3, KRTHB1, LY6E and RARRES3 as well as upregulation of genetic inhibitors of lung metastasis such as deleted in liver cancer 1 (DLC1) and progestagen-associated endometrial protein, PAEP (Dydensborg et al., 2009). GATA3 may also suppress metastasis *via* the reversal of EMT (Yan et al., 2010).

P63

The *p63* gene, a homologue of the tumor suppressor *p53*, is highly expressed in the basal or progenitor layers of epithelial tissues. Very strikingly, *p63*$^{-/-}$ embryos fail to induce all MRs and all other ectodermal appendages (Mills et al., 1999; Yang et al., 1999).

P63 has multifaceted roles in breast cancer (Gatti et al., 2019). Notably, the overexpression of *H-Ras*^{V12} or *PIK3CA*^{H1047R} oncogenes in MCF10A and MCF12A normal breast cell lines downregulates the expression of Δ*Np63*, a *p63* isoform, which leads to EMT, increased cell motility, and invasiveness. Importantly, silencing of Δ*Np63* alone induces EMT and recapitulates the pro-migratory action of these oncogenes; highlighting Δ*Np63* as a critical effector (Yoh et al., 2016). The invasive properties of *p63* may be mediated through its target gene, membrane-type 1 membrane-anchored matrix metalloproteinase (MT1-MMP), a protease that is upregulated in *p63*-high basal breast cancers (Lodillinsky et al., 2016).

Δ*Np63* is also involved in controlling the self-renewal potential and expansion of mammary CSCs. Downregulation of *p63* in MMTV-ErbB2-derived mammospheres significantly limits its self-renewal capacity *in vitro* and delays tumor growth *in vivo*. At the molecular level, Δ*Np63* enhances HH signaling by directing the expression of SHH, GLI2, and PTCH1 (Memmi et al., 2015).

Hormone Signaling

The steroid hormones estrogen and progesterone are essential for the development and function of the breast, bone as well as the reproductive and cardiovascular systems. Classically, estrogen or progesterone binding to their cognate nuclear receptors leads to receptor dimerization, nuclear translocation and binding to DNA response elements to activate or inhibit target gene expression (Fuentes and Silveyra, 2019) (**Figure 3I**).

Hormone signaling is essential for sexual dimorphism, a process where MR development diverges between the two sexes in the embryonic mouse MRs at E14.5. In males, testosterone acts on the AR-expressing mesenchyme to constrict the mammary epithelium which halts further development (Durnberger and Kratochwil, 1980; Sakakura et al., 1982). When AR expression is absent or downregulated, MR development proceeds aberrantly (see sections on BMP, PTHRP, P190B RHOGAP, IRS, and IGF1R signaling).

Hormone receptor (ER or PR)-positive breast cancers constitute the major proportion of breast cancer subtypes. The transcriptional activity of ERα is regulated by its post-translational modifications and the action of nuclear receptor co-regulators, which may contribute to the development of breast cancer (Manavathi et al., 2013). The cross-talk of ER with other steroid receptors like PR affects tumor progression. Notably, progesterone may enhance the anti-proliferative effect of standard anti-estrogen therapy by influencing ER binding and its target gene transcription (Siersbaek et al., 2018).

ERα signaling has been implicated in metastasis. In ERα^{+} primary tumors, more than 80% of lymph node metastases, and 65–70% of overall distant metastases retain ERα expression. Moreover, ERα expression in tumors is also correlated with the development of bone and lung metastases (Saha Roy and Vadlamudi, 2012). Mechanistically, the bone tropism of metastatic breast cancer cells may be mediated by the interaction of ERα and the EMT transcription factor ZEB1, which have been shown to modulate ERα-mediated transcription induced by estrogen or cAMP signaling (Mohammadi Ghahhari et al., 2022). ERα knockdown in MCF7 breast cancer cells induces potent EMT and changes in the expression and activity of matrix macromolecules (Bouris et al., 2015). Functional cross-talks between estrogens and insulin/insulin-like growth factors (IIGFs)—by affecting the tumor microenvironment—may contribute to metastasis (Vella et al., 2020).

In the clinic, anti-estrogen therapy including selective ER modulators (SERMs), selective ER down-regulators (SERDs), and aromatase inhibitors (AIs) is the standard of care for patients with ERα^{+} breast cancers (Siersbaek et al., 2018).

Evidently, the preceding list of genes and signaling pathways have demonstrably clear, important roles in embryonic mammary gland development and often context-dependent roles in promoting breast cancer. GATA3 mutations are almost always associated with breast cancer compared to other cancers. Similarly, PTHRP signaling dysregulation is frequently implicated in breast cancer and bone metastasis. As a hormone-sensitive tissue, hormone signaling dysregulation has important implications for breast tissue. This, however does not imply that these genes and signaling pathways have exclusive roles or specific associations with breast cancer. Indeed, many are known to drive tumorigenesis in other tissues in different contexts. In the case of WNT signaling, loss-of-function mutations in APC were first implicated in the hereditary colon cancer syndrome, familial adenomatous polyposis whereas HH signaling dysregulation is predominantly known to drive basal cell carcinomas. These and examples of other cancers are listed in **Table 1**.

PARALLELS OF PROPERTIES AND MOLECULAR SIGNATURES OF EMBRYONIC CELLS IN BREAST CANCER

Cellular Lineages and Stem Cell Potency

The cellular hierarchy and clonal dynamics of cells during pre- and postnatal mammary gland development have been greatly clarified by *in vivo* lineage tracing methodologies. Promoter specific-Cre models that activate the expression of a reporter gene encoding a fluorescent protein or *lacZ* enable the identification and facilitate the tracking of progenies from a defined parental cell (Kretzschmar and Watt, 2012).

By and large, *in vivo* lineage tracing studies with multiple promoter-Cre models suggest that during the initial stages of development, embryonic mammary cells are multipotent at the population level (Van Keymeulen et al., 2011; Rodilla et al., 2015; Trejo et al., 2017; Wuidart et al., 2018). Clonal analyses, where a small number of cells (<1%) is initially labelled, showed the progressive segregation of basal and luminal lineages and has revealed of unipotent luminal cells such as *Notch1*^{pos} cells at E12.5 (Lilja et al., 2018). Other subsets of unipotent luminal cells include *Axin2*-expressing cells at E12.5 (Van Amerongen et al., 2012) and the zinc finger transcriptional repressor, *Blimp1*-expressing cells at E17.5 (Elias et al., 2017). During postnatal development, evidence for both unipotency and multipotency the mammary stem epithelia have been reported. While overwhelming evidence suggest the unipotency and lineage restriction of basal and luminal cells through *in vivo* lineage tracing (Van Keymeulen et al., 2011; de Visser et al., 2012; van Amerongen et al., 2012; Lafkas et al., 2013; Prater et al., 2014; Rodilla et al., 2015; Trejo et al., 2017; Lilja et al., 2018; Wuidart et al., 2018; Matsuo et al., 2022), rare bipotent basal cell clones expressing *Krt5*, *Krt14*, *Lgr5* (Rios et al., 2014), the WNT target, *Procr* (Wang et al., 2015) and the NOTCH ligand *Dll1*-expressing cells (Chakrabarti et al., 2018) have also been observed.

During postnatal development, it is postulated that luminal and basal cell interactions are crucial to maintain lineage fidelity. Specifically, cell-cell interactions are mediated by TNF which is secreted by luminal cells and restricts basal cell multipotency. Ablation of luminal cells in the adult mammary gland reactivates the multipotency of normally unipotent basal stem cells both *in vivo* and in organoid models. Bulk- and single-cell transcriptomic analyses reveal that after luminal cell ablation, basal cells activate a hybrid basal and luminal cell differentiation program that is reminiscent of the genetic program that regulates multipotency during embryonic development before giving rise to luminal cells. This multipotency program is mediated by the activation of NOTCH, WNT and EGFR and downregulation of the TNF signaling pathways. Therefore, NOTCH, WNT and EGFR pathway inactivation, or TNF pathway activation were able to inhibit regeneration-induced basal cell multipotency. This demonstrates that heterotypic communication between luminal and basal cells—tightly regulated by embryonic pathways such as NOTCH and WNT—is essential to maintain lineage fidelity and stem cell potency in mammary epithelial stem cells (Centonze et al., 2020). It is tempting to speculate that disruption of normal cell-cell interactions that lead to a reversion to a multipotent embryonic cell state may be an early event in tumorigenesis. Similar to lineage ablation, reprogramming of cell states by an oncogenic stimulus such as PIK3CA^{H1047R} results in the acquisition of multipotency of both basal and luminal cells in the postnatal mammary gland and the recapitulation of embryonic gene signatures (Wuidart et al., 2018). This, in turn, leads to the development of different breast cancer subtypes and the acquisition of tumor heterogeneity (Koren et al., 2015; Van Keymeulen et al., 2015).

Embryonic Molecular Signatures in EMT, Stem Cells and Breast Cancer Subtypes

Expression profiles analyses of various human tumor types have revealed the enrichment patterns of gene sets associated with embryonic stem cell identity. In breast cancers, this embryonic stem cell-like signature is often associated with high-grade, basal subtype ER^{-} tumors, with poor clinical prognosis (Ben-Porath et al., 2008).

Several recent studies have harnessed the advancements of multi-omics technologies to investigate the presence of embryonic signatures in breast tumor models. Bulk transcriptomic analyses show that subsets of embryonic mammary epithelial signature at E12.5 are activated in mouse *Brca1*$^{-/-}$;*p53*$^{+/-}$ tumors and malignant human basal-like breast cancers. The signature is composed of genes that encode predominantly transcriptional regulators, notably *Hox* genes, cell cycle, and actin cytoskeleton components. There is also evidence that the embryonic signatures that are reactivated in cancers are subtype-specific. Embryonic gene subsets that include regulators of neuronal differentiation, transcription, and cell biosynthesis were enriched in non-basal-like tumor subtypes and repressed in basal-like tumors. Moreover, several embryonic genes showed significant upregulation in hormone receptor negative, and/or grade 3 breast cancers. Notably, the transcription factor, SOX11, a progenitor cell and lineage regulator of non-mammary cell types, is found to be highly expressed in some *Brca1*$^{-/-}$ mammary tumors. Cancer cells may also activate latent embryonic mesenchymal signatures to undergo EMT. A list of 25 genes—ATL3, B3GNT5, BCL11A, CDCA2, CHST2, CORO1C, DNM1L, DNMT3A, EPHA4, GPC2, HDGF, IGF2BP3, JMJD4, KIF20A, PROX1, PTDSS1, RPS6KA3, SLC16A13, SOX11, TCF7L2, TMEM38A, TMOD1, TRIB2, TTC9C, and UCHL1—were found in the 37-gene tumor-associated embryonic epithelial signature. This gene set could be further evaluated for their roles as putative regulators of EMT in breast cancers and potentially serve as new targets for therapeutic intervention in the future (Zvelebil et al., 2013).

There is evidence for significant molecular similarity of stem-like subpopulations of mammary cells which are enriched at E18.5 (Spike et al., 2012; Makarem et al., 2013; Trejo et al., 2017), to breast cancers. Specifically, the fetal mammary stem cell (fMaSC) signature was enriched among aggressive basal-like and Her2^{+} tumors. The co-expression of myoepithelial and luminal keratins as well as vimentin, which is characteristic of the fMaSC-like state, suggests that the reversion of cancer cells to an embryonic-like state resembling the fMaSC and/or fetal stroma (fSTR) compartments could be driven by partial EMT

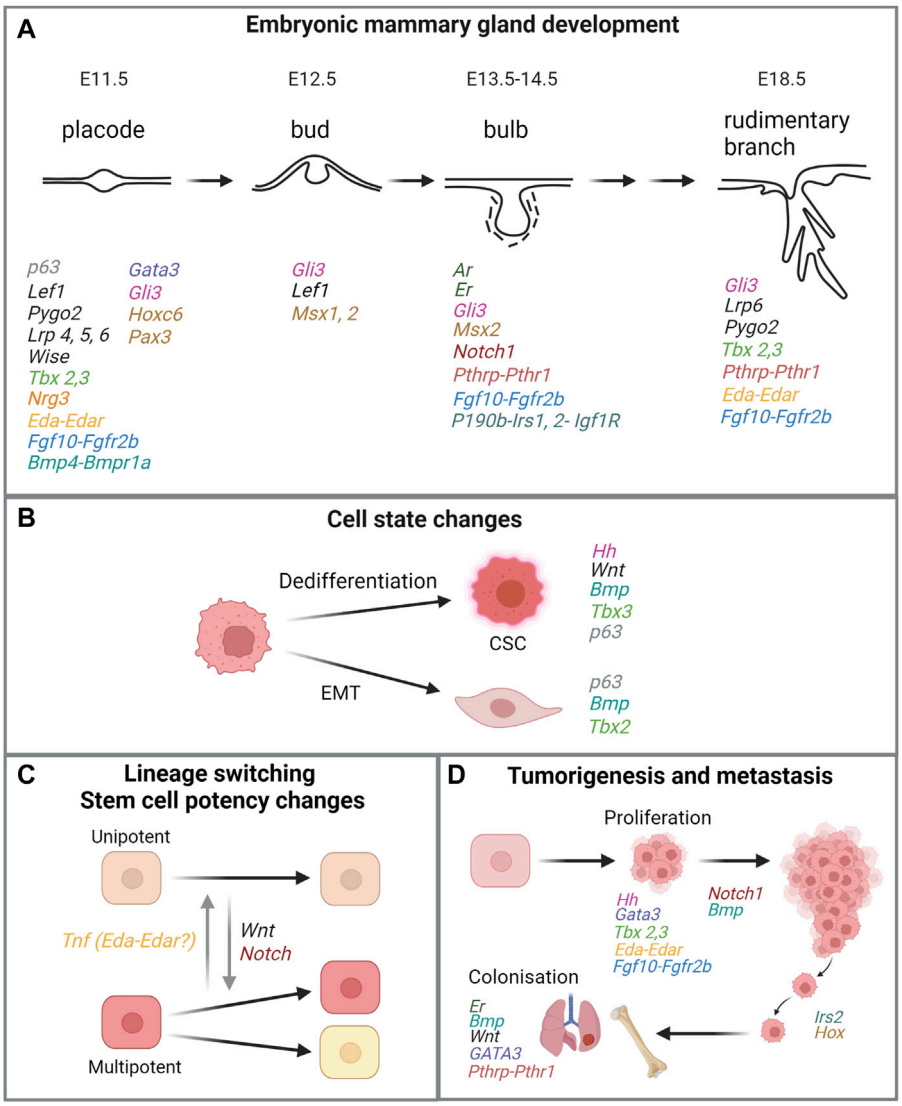


FIGURE 4 | Similarities of genes or signaling pathways critical in embryonic mammary gland development and processes in breast cancer. **(A)** Key stages in embryonic mammary gland development and genes and their associated pathways involved in the respective stages. For MR-specific developmental processes that are regulated by relevant genes and signaling pathways, please refer to main text. **(B,C,D)** Processes that are linked to tumorigenesis and metastasis and mediated by the same genes or signaling pathways important for embryonic mammary gland development. Genes in the same signaling pathway are coded in the same colour. Figure created with BioRender.

(Spike et al., 2012). On the other hand, a stem-like, quiescent, hormone-sensitive subpopulation of mammary gland cells which originate in the MRs, *Lgr5*$^{+}$*Tspan8*^{hi}, has been shown to bear molecular features of claudin-low breast tumors (Fu et al., 2017).

In a follow-up to the Spike (2012) study using single-cell transcriptomic analyses, Her2^{+} tumors and basal-like tumors - but not the equally proliferative luminal B tumors—were found to frequently show enrichment of fMaSC-like metabolic profiles including glycolysis, the Krebs cycle and fatty acid oxidation. (Giraddi et al., 2018). This finding suggests that the re-emergence of embryonic metabolic programs could be less associated with the enhancement of tumor cell proliferation. Rather, other processes associated with tumorigenesis such as cell state changes, cellular plasticity, and lineage specification, could be directed by such embryonic programs (Ben-Porath et al., 2008).

In silico analyses comparing bulk TCGA tumor gene expression data to the Giraddi mouse developmental trajectory show that normal, luminal A, and luminal B tumors map most closely to mature adult cells whereas Her2^{+} tumors map to slightly more immature cells. Basal tumors, which harbour great molecular heterogeneity, spanned the pseudotime encompassing both embryonic and adult cells along the basal trajectory (Thong et al., 2020). In the same study, gene expression analyses revealed that normal human mammary (NM) cells aligned to adult mouse cells whereas NM cells, which are conditionally-reprogramed (CR) *in vitro* to promote stem-like features,

aligned more closely with mouse embryonic cells across the pseudotime trajectory. In contrast to NM cells, CR cells also develop hybrid cell states, characterized by the co-expression of basal and luminal lineage markers or epithelial and mesenchymal markers that are associated with aggressive cancers. Taken together, these results suggests that the acquisition of embryonic programs converts cells into a stem-like state, presenting with characteristics typical of a more developmentally immature phenotype.

There is also evidence that metastatic cells may leverage on embryonic or more generic pluripotency programs to facilitate malignant progression. Single-cell analyses show that low-burden metastatic cells harbouring basal- or stem-like characteristics may exploit embryonic programs for self-renewal and maintenance by the upregulation of pluripotency genes POU5F1 (also known as OCT4) and SOX2 as well as classical EMT markers (Lawson et al., 2015).

EMBRYONIC MOLECULAR SIGNATURES AND THEIR CLINICAL RELEVANCE FOR BREAST CANCER

Breast cancer and metastasis remain challenging problems globally. The striking similarities between embryonic progenitor cells to breast cancers and metastases could pave the way to pinpoint novel prognostic markers and targets for therapeutic intervention (**Figure 4**). It has been proposed that critical signaling pathways that promote pathogenesis may be masked by the over-representation of proliferation-, ER-, and Her2-related signaling signatures in many existing prognostic signatures. Therefore, these minor but critical signaling pathways may be uncovered by studying and distilling normal developmental paradigms such as the fMaSC and fSTR states. Such approaches could potentially deconvolute the complexities of tumor heterogeneity by narrowing the focus on subpopulations of cells that exhibit embryonic signatures and identifying targets specific for these populations.

There are some indications that show embryonic signatures may have clinically-relevant prognostic value. Only a very low percentage of patients who are treated with neoadjuvant chemotherapy will progress to pathological Complete Response (pCR). For this reason, novel approaches that can estimate the probability of pCR are highly desired. The predictive value of normal cellular expression features for pCR was evaluated using univariate and multivariate logistic regression analyses. Indeed, human luminal progenitors (LumProg) and mouse fMaSC expression features were identified as predictive of neoadjuvant chemotherapy efficacy across all breast cancer patients. These signatures were highly expressed in basal-like tumors, consistent with the clinical observation that basal-like tumors have better neoadjuvant chemotherapy response rates. On the other hand, benign luminal A and B tumors which are typically more resistant to neoadjuvant chemotherapy exhibit high expression of another MaSC signature subset (fMaSC-MmEnriched-feature2). Importantly, the prognostic value of these normal and embryonic signatures remained significant even after accounting for tumor intrinsic subtype, proliferative status, and other clinical parameters; in other words, normal cell signatures add information and prognostic value that are distinct from clinical features (Pfefferle et al., 2015). Along a similar vein, a prognostic gene expression signature derived from the E6.5 mouse which is representative of extensive cellular plasticity was shown to predict metastatic competence in human breast tumor cells (Soundararajan et al., 2015).

CONCLUSION AND PERSPECTIVES

The misregulation of genes and signaling that are important in regulating normal embryonic mammary gland development frequently occur in pathological conditions such as breast cancer (Howard and Ashworth, 2006). Thus, the identification and study of the mechanisms mediated by genes and signaling pathways during early development may give rise to deeper understanding of disease mechanisms. Notably, embryonic molecular signatures may complement conventional clinical parameters for the stratification of patients and to offer accurate predictions of outcomes among breast cancer patients.

To date, practical considerations and sample limitations have resulted in pooled mammary gland molecular profiling. Considering the unique genetic and developmental history of the mammary glands, such data pooling may have implications in the success of cancer research. Thus, it may be prudent to make MR-specific comparisons and analyses in future research.

Likewise, it will be of interest to further characterize and locate cells harbouring different cell states such as the hybrid state, reminiscent of embryonic cells, in the adult mammary gland and tumors using techniques such as spatial transcriptomics. With recent technical advancements in a suite of multi-omics methods, the identification of the metabolomic and epigenomic states of the embryonic mammary glands—and the parallels to breast cancers - will be exciting themes of research that will illuminate more insights into mammary gland development and cancer pathogenesis.

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MYL wrote the manuscript.

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GLOSSARY

Mammary rudiment (MR) The mammary gland during embryogenesis.

Placode Mammary rudiment at around E11.5 characterized by a multi-layered, disk-shaped morphology.

Genetically engineered mouse model (GEMM) Mouse (Mus musculus) with its genome modified using genetic engineering techniques. Genetic modifications may include gene loss-of-function (knockout, hypomorph) or gain-of-function (overexpression).

Sexual dimorphism Developmental process in the E14.5 mouse where development diverges in male and female.

Epithelial-to-mesenchymal transition (EMT) A process that enables cancer cells to suppress their epithelial features and take on mesenchymal ones. As a result, cells detach from their primary site and migrate to distal sites.

Cancer stem cell (CSC) Subpopulation of cancer cells with tumor-generating and multi-lineage differentiation potential. These cells are commonly associated with chemoresistance.

MMTV-PyMT (mouse mammary tumor virus-Polyoma virus middle T antigen) Mouse mammary gland carcinoma model, which is characterized by rapid development of multifocal tumors and extensive lung metastasis.

Lineage tracing A method to study the progenies of an initial population of cells that are marked genetically by a promoter-specific-Cre construct in combination with a reporter gene such as GFP.

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Asymmetric Cell Division and Tumor Heterogeneity

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Asymmetric cell division (ACD) gives rise to two daughter cells with different fates after mitosis and is a fundamental process for generating cell diversity and for the maintenance of the stem cell population. The cancer stem cell (CSC) theory suggests that CSCs with dysregulated self-renewal and asymmetric cell division serve as a source of intra-tumoral heterogeneity. This heterogeneity complicates the diagnosis and treatment of cancer patients, because CSCs can give rise to aggressive clones that are metastatic and insensitive to multiple drugs, or to dormant tumor cells that are difficult to detect. Here, we review the regulatory mechanisms and biological significance of asymmetric division in tumor cells, with a focus on ACD-induced tumor heterogeneity in early tumorigenesis and cancer progression. We will also discuss how dissecting the relationship between ACD and cancer may help us find new approaches for combatting this heterogeneity.

Keywords: asymmetric cell division, tumorgenesis, drug resisitance, symmetric cell division, tumor heterogeneity

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INTRODUCTION

Stem cells are known for their ability to self-renew and to differentiate into different cell types (Morrison and Kimble, 2006). One strategy by which stem cells achieve these two goals is through a unique mode of division: stem cells can either replicate itself through symmetric cell division (SCD) or produce two daughter cells with different cell fates through asymmetric cell division (ACD) (Kahney et al., 2017). A balance between these two forms of division is essential for maintaining tissue homeostasis; failure to maintain homeostasis can lead to severe outcomes such as tumorigenesis (Neumüller and Knoblich, 2009).

Cancer stem cells (CSCs), a subpopulation of tumor cells that possess stem cell-like properties, have been identified in many tumor types. Accumulating evidence suggests that CSCs with dysregulated self-renewal and ACD give rise to tumor cells with a variety of properties and thus serve as a source of intra-tumoral heterogeneity (Lee et al., 2016). This heterogeneity complicates the diagnosis and treatment of cancer patients, because CSCs can generate tumor cell clones that are multi-drug-resistant, metastatic, or dormant, which makes them difficult to detect (Knoblich, 2010; Singh and Settleman, 2010; Viale et al., 2014). Here, we review the current understanding of ACD and discuss the relationship between ACD and tumor heterogeneity.

MECHANISMS OF ASYMMETRIC CELL DIVISION

The basic mechanisms of ACD were initially explored in *Drosophila melanogaster*. The construction of the *Drosophila* nervous system is mediated by embryonic neuroblasts (NBs) through a series of ACD events: first of all, cellular components in NBs are distributed asymmetrically before mitosis, cell-fate-determining factors such as Numb、Brat (TRIM32 in vertebrates) and Prospero (PROX1 in vertebrates) are concentrated in the basal cell cortex, while the apical region expresses strong stemness signals, leading to unequal separation during cytokinesis (Bello et al., 2008; Bowman et al., 2008). As a result, ACD of NBs produces another NB and a more differentiated progenitor cell called ganglion mother cell (GMCs). Studies in *Drosophila* have also uncovered proteins involved in the establishment of cell polarity, including the polarity complex Par3/Par6/aPKC and the related protein WD40 protein lethal giant larvae (Lgl) (Lee et al., 2006a), as well as protein kinase Aurora-A (Wirtz-Peitz et al., 2008). Proteins necessary for polar coupling of mitotic spindle to the cell cortex have been identified, such as Pins (LGN in vertebrates), the Par3 binding protein Inscuteable, the heterotrimer G protein subunit Gαi and the Dynein adapter Mud (NuMA in vertebrates) (Izumi et al., 2006; Santoro et al., 2016). Insights into the molecular mechanisms of ACD in the *Drosophila* model warrant further studies in vertebrates.

In vertebrates, molecular determinants of ACD are highly conserved; however, their modes of division may vary depending on the cell/tissue type. In recent years, advancements in research technologies, such as stem cell cultures, lineage tracing, cell imaging and molecular tracers, have largely facilitated the study of stem cell ACD in more complex mammalian systems, providing insights into the complexity of cellular and environmental asymmetry that play important roles in cell fate determination (Santoro et al., 2016). Studies so far suggest that ACD in mammalian stem cells is mediated by two different mechanisms: one is niche-dependent ACD, which is induced by external signals; the other is termed spontaneous ACD, which is determined by the differential distribution of proteins, RNA transcripts and macromolecules between two daughter cells.

Multiple studies have revealed that the Notch signaling pathway plays a central role in instructing stem cell ACD (Srinivasan et al., 2016a; Rossi and Desplan, 2017). Numb negatively regulates Notch signaling, the asymmetric distribution of Numb is regulated by the Par3/Par6/aPKC complex; the assembly of the Par3/Par6/aPKC complex alters the substrate specificity of aPKC to phosphorylate Numb, resulting in its release from the apical cortex. Phosphorylated Numb is localized to the basolateral cell cortex with the help of an adapter protein, partner of Numb (PON), which is activated by the Polo kinase (Gómez-López et al., 2014). Notch signaling is restricted by the asymmetric distribution of Numb; Numb mediates ubiquitin-dependent degradation of the Notch receptor and blocks the nuclear translocation of Notch intracellular domain (NICD1) (Mcgill and Mcglade, 2003). In addition, studies have shown that the microRNA miR-34, suppresses Notch expression by directly binding to the 3′ untranslated region (UTR) of Notch mRNA; the asymmetric distribution of miR-34 results in distinct cell fates in the two daughter cells, acting as a bimodal switch between self-renewal and differentiation. Interestingly, miR-34 also inhibits Numb expression by binding to the 3′UTR of Numb mRNA. Thus, miR-34, Numb, and Notch form an incoherent feedforward loop (IFFL), which maintains the homeostasis of Notch level (Bu et al., 2016) (**Figure 1A**). This regulatory mechanism further fine-tunes Notch signaling and cell fate determination.

Wnt signaling is another key regulator of stem cell ACD (Varga and Greten, 2017). A Wnt ligand gradient in the stem cell niche has been shown to instruct the directed movement of centrosomes and mitotic spindles and mediate the differential distribution of downstream Wnt signaling molecules in daughter cells (**Figure 1A**). The daughter cell proximal to higher levels of Wnt expresses high levels of Wnt pathway genes including β-catenin and stemness-related genes, thereby maintaining the stemness of the daughter cell. On the contrary, the daughter cell distal to Wnt is destined to differentiate. ACD guided by Wnt in the stem cell niche ensures the orderly spatial distribution of stem cells and differentiated progenies.

Another molecular determinant of ACD is p53 (Santoro et al., 2016), a well-studied tumor-suppressor that induces cell-cycle arrest and apoptosis in cells with DNA mutations or damage. Importantly, p53 is involved in the maintenance of the stem cell pool by regulating the modality of cell division. In mammary gland epithelial cells, p53 expression induces a shift from exponential growth to linear growth, restricting the expansion of mammary epithelial cells by upregulating the ratio of stem cells that undergo ACD (Cicalese et al., 2009). Furthermore, Numb interacts with and stabilizes p53 by blocking the ubiquitination and degradation of p53 induced by the E3 ubiquitin ligase MDM2 (Tosoni et al., 2015; Kim and Ronai, 2018) (**Figure 1A**).

ASYMMETRIC CELL DIVISION AND TUMOR HETEROGENEITY

Asymmetric Cell Division and Cancer Stem Cell Heterogeneity in Tumorigenesis

In the early stages of tumorigenesis, normal cells acquire key driver mutations that confer a growth advantage, and undergo rapid clonal expansion (Gerdes et al., 2014). It has been shown that the aberrant expression of key regulators of ACD is an important contributor to early carcinogenesis. When NBs carrying loss-of-function mutations in key regulators of ACD were allografted into the abdomen of wild-type adult *Drosophila*, a rapid expansion and invasion of the mutant cell population into the host's abdomen was observed; and the tumorigenicity of the mutant cells increased with subsequent passaging of the allografts, suggesting that uncontrolled proliferation due to dysregulation of ACD was one of the key factors in the tumorigenic transformation (Lee et al., 2006a; Betschinger et al., 2006; Lee et al., 2006b; Bowman et al., 2008). In mammalian models, a switch from ACD to SCD in stem cells triggers a severe disruption of tissue homeostasis and drives

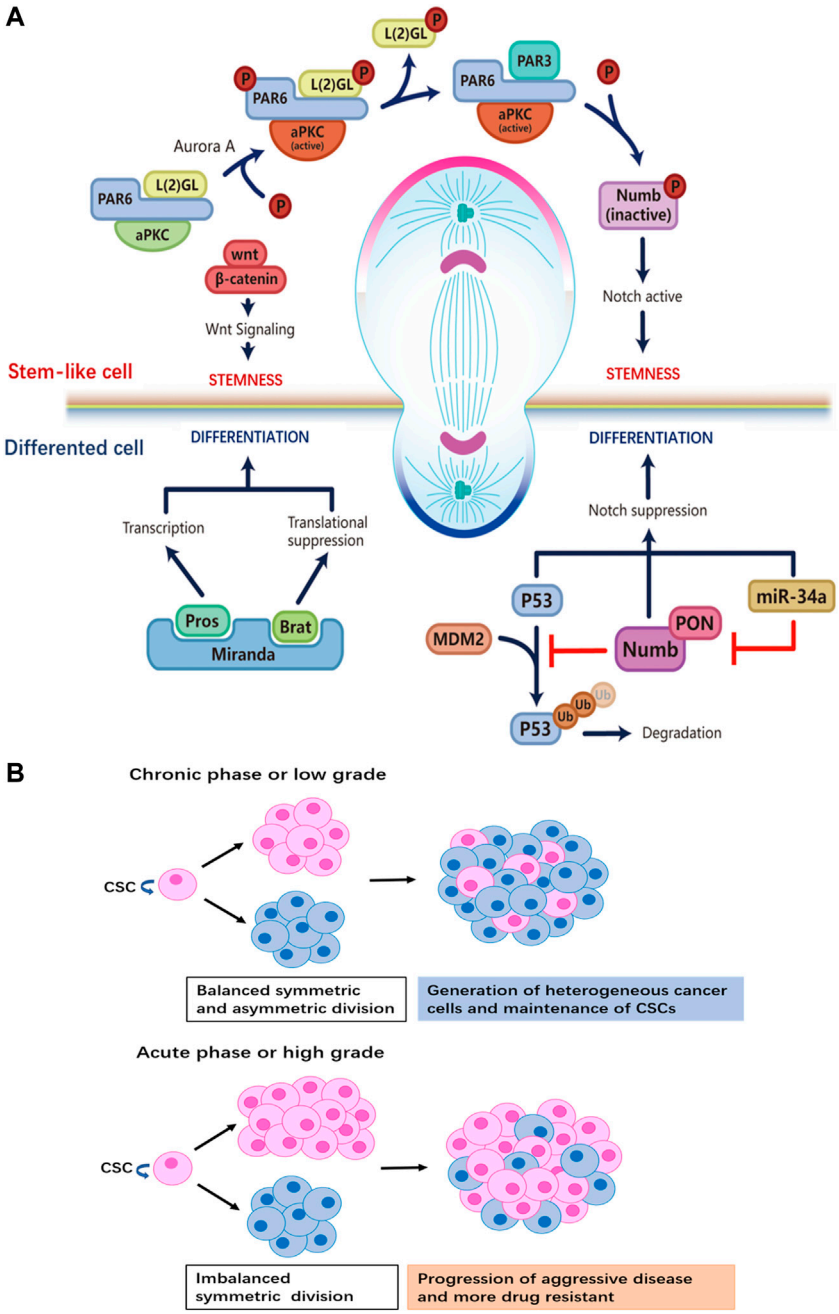

FIGURE 1 | (A) Distinct regulatory machineries are involved in the two daughter cells during ACD of stem cells. Stem cell at the apical pole: formation of the aPKC/PAR6/PAR3 complex plays a crucial role for the establishment of the 'self-renewal' identity at the apical pole. Aurora-A protein kinase activates aPKC, which then phosphorylates L (2)GL; then L (2)GL is released from the complex and replaced by PAR3. The aPKC/PAR6/PAR3 complex phosphorylates Numb, releasing it from the apical membrane. PON brings Numb to the basal pole that will become the differentiated daughter cell. By removing Numb, Notch signaling is active to maintain the stemness of the daughter cell at the apical side. Wnt signaling also participates in promoting self-renewal in the daughter cell, though details of the mechanism are not known. Differentiated cell at the basal pole: the adapter protein Miranda recruits Prospero and Brat. As a translational repressor, Brat suppresses the synthesis of proteins necessary for proliferation. The transcription factor Prospero promotes the expression of genes that drive differentiation after Miranda is degraded. Ubiquitination-dependent degradation of P53 is inhibited by Numb, while the miR-34-Numb-Notch feedback loop suppresses Notch level and favors differentiation in the daughter cell. **(B)** The relationship between tumor progression and the ratio of CSCs that undergo ACD versus SCD. When SCD and ACD are balanced, tumor generates heterogeneity while maintaining the pool of CSCs. On the other hand, a switch from ACD to SCD in CSCs results in the expansion of the stem cell pool.

tumor formation (Klezovitch et al., 2004; Mccaffrey and Macara, 2009); regulatory factors of ACD are frequently found in lists of aberrantly expressed genes associated with cancer (Pece et al., 2004; Ito et al., 2010; Gómez-López et al., 2014). However, mutating this factor alone is not sufficient for tumor initiation in mammals (Iden et al., 2012; Mccaffrey et al., 2012).

TABLE 1 | ACD-related regulators and their role during cancer formation and progression.

Gene	ACD-related Pathway	Model	cancer Type	Dysregulation in Cancer	Influence on the division mode
NUMB-interacting protein (TBC1D15)	Numb-Notch1-Nanog、P53	mouse	Hepatocellular carcinoma (HCC)	overexpressed	promote symmetric renewal、promote stemness
MAD2	-	mouse	gastric carcinoma (GC)	overexpressed	promote stemness
Lnc34a	Notch、Wnt	mouse	colon cancer	overexpressed	promote symmetric renewal
CDC42	Wnt	mouse	triple receptor negative breast cancer (MDA-MB-231 cell line)、blood/acute myeloid leukemia	overexpressed	promote ACD
Aurora-A	Numb-Notch、p53	*Drosophila*	-	overexpressed	maintaince ACD、promote stemness
miR-34a	Numb-Notch	mouse	colon cancer	decreased expression	promote differentiation
CD44/Zeb1 loop	-	mouse	lung adenocarcinoma	express in cancer-generating cells	promote ACD
SOX2	-	mouse	Head and Neck Squamous *Cancer* Stem Cells	critical for propagation of CSCs	maintaince ACD
EGFL6	-	mouse	ovarian cancer	express in tumor vascular cells and in some cancer cells	induce ACD
PKCι	Notch	mouse	lung adenocarcinoma	overexpressed	drive ACD
APT1	Numb-Notch、wnt	mouse	MDA-MB-231 cell line	critical for propagation of CSCs	direct ACD
lis1	Numb-Notch	mouse	acute myelogenous leukemia (AML)	critical for propagation of CSCs	promote symmetric renewal
miR-200b-3p	Notch	mouse	pancreatic cancer	decreased expression	promote ACD
APC	Wnt/β-catenin	mouse	colon cancer	frequently mutated	direct ACD
numb	Numb-Notch	mouse	mammary carcinomas, lung cancer, chronic myeloid leukemia	decreased expression	promote differentiation
p53	Numb-Notch	mouse	Breast cancer	decreased expression	promote ACD

It has been hypothesized that certain tumors originate from normal stem cells (Sell, 2007), but the mechanisms by which normal stem cells progressively undergo malignant transformation are not clear (Kasper, 2008; Sell, 2010). Recently, researchers have identified a link between the dysregulated division pattern of +4 stem cell (SC) in the gastric antrum and gastric carcinogenesis. +4 SCs, marked by expression of Cck2r [a G-protein coupled receptor (GPCR)] and Delta-like ligand 1 (DLL1), are $Notch^{low}/Numb^{+}$ cells that undergo ACD predominantly under normal conditions; and their proliferation is inhibited by signaling from gastrin-expressing endocrine cells. Studies in mouse models have shown that treatments with carcinogens lead to a down-regulation of gastrin secretion and as a result, + 4 SCs gradually up-regulate the proportion of cells that undergo symmetric division, leading to an expansion of the + 4 SC pool. The disruption in tissue homeostasis caused by the switch to symmetric division is thought to be closely related to gastric carcinogenesis (Chang et al., 2020). Another study has shown the involvement of ACD in early tumor formation in mutant K-Ras-induced spontaneous lung cancer model. The pre-cancerous adenoma cells initiated a positive CD44/Zeb1 feedback loop through nuclear polarization of key transcription factors during asymmetric division, generating an intermediate transitional population of $Zeb1^{hi}CD44^{hi}$ cells that are tumorigenic (Liu et al., 2018).

CSCs have been reported to control the ratio of cells that undergo ACD versus SCD during early tumorigenesis. In order to achieve rapid clonal expansion and establish survival advantage, CSCs enable self-renewal at a certain rate to preserve stemness, while generating differentiated cells to constitute a heterogeneous tumor. **Table 1** summarizes recent reports on how ACD-related pathways/genes influence cancer progression (Zimdahl et al., 2014; Tosoni et al., 2015; Ali et al., 2016; Bai et al., 2016; Damodaran et al., 2017; Keysar et al., 2017; Mizukawa et al., 2017; Castro-Oropeza et al., 2018; Liu et al., 2018; Stypulkowski et al., 2018; Sugioka et al., 2018; Choi et al., 2020; Pajuelo-Lozano et al., 2020). Interestingly, the regulatory factors of ACD display heterogeneous expression: factors that direct the differentiation of daughter cells are often associated with loss-of-fuction mutations or downregulation; while factors that maintain the stemness are often overexpressed in tumors. These factors affect ACD and cell heterogeneity directly or indirectly through Notch or Wnt signaling pathway. For example, the palmitoacylase APT1 promotes the asymmetric localization of Numb and β-catenin on the plasma membrane by interacting with CDC42. APT1 contributes to the activation of Notch or Wnt signaling, while CDC42 restricts APT1 activity to only one of the two daughter cells, results in formation of heterogeneous cell population during ACD. APT1 and CDC42 cooperate to maintain a self-renewal stem cell pool; and loss of APT1 depletes a specific tumorigenic stem cell subpopulation (Stypulkowski et al., 2018). Another

study has shown that in KRAS-mediated lung adenocarcinoma (LADC), the protein kinase Cι(PKCι) activates NOTCH3 expression by phosphorylating ELF3, which driving ELF3 recruitment to NOTCH3 promoter. The unequal distribution of PKCι results in a difference in NOTCH3 signaling levels between two daughter cells. This difference ultimately leads to ACD of tumor-initiating cells (TICs), preserving the TIC pool while generating heterogeneous populations (Ali et al., 2016). These discoveries suggest that ACD plays an important role in cell heterogeneity and cancer progression.

Asymmetric Cell Division and Cancer Heterogeneity in Drug Resistance

CSCs are capable of responding rapidly and flexibly to environmental challenges, making CSCs a major source of drug-resistant tumor cells that give rise to disease recurrence after drug treatment (Cabrera et al., 2015; Batlle and Clevers, 2017). The transition of CSCs from asymmetric to symmetric renewal division increases the proportion of CSCs in a tumor, which is predictive of malignant progression of disease and increased difficulty in treatment (Lytle et al., 2018). Aside from being able to swiftly adapt and react to various external stresses, CSCs pose challenges to cancer therapy as they are a source of high intra-tumoral heterogeneity (Singh et al., 2015), which leads to different sensitivity to treatment in cancer patients (Donnenberg and Donnenberg, 2005) (**Figure 1B**).

It has been shown that tumor cells can asymmetrically divide to produce progenies that are slow-cycling and those that are fast-cycling. Fast-cycling cells rapidly expand to accelerate tumor progression, while slow-cycling cells have a relatively slower doubling rate and may survive chemotherapy targeting the rapidly proliferating population, therefore serving as an important reservoir of tumor-initiating cells post-treatment. Studies have demonstrated that after ACD, CSCs can produce dormant cells that retain labeled nucleotide or fluorescent lipid markers (Pece et al., 2010; Majumdar et al., 2020). In patients with breast cancer, ACD of fast-cycling cancer cells produced slow-cycling G0-like progenies that are $AKT^{low}Ros^{low}Hes1^{hi}$, in a process that is dependent on the asymmetric inhibition of AKT/PKB kinase signals in the two daughter cells at the end of mitosis (Dey-Guha et al., 2011). In colorectal cancer (CRC), a subpopulation of tumor cells with stem cell properties, called colorectal cancer-initiating cells (CCIC), harbors high internal heterogeneity. There are two types of CCICs ——MYC-dependent, fast-cycling cells expressing LGR5, CD133, and CD44, and slow-cycling cells expressing BMI1, hTERT, and HOPX. It was found that the 2 cell populations could be transformed into each other by ACD (Srinivasan et al., 2016b). Interestingly, compared to the one-way transition from fast-cycling cells to slow-cycling cells in breast cancer, ACD in colorectal cancer establishes a bi-directional transition between the 2 cell populations, thereby sustaining both growth potential and drug resistance of the tumor, enabling rapid response and adaptation of the tumor to a dynamic environment.

Furthermore, tumor cells can generate progenies with survival advantages through ACD, by the selective enrichment of factors that are pro-survival to one of the daughter cells (Lytle et al., 2018). For example, the ATP-binding cassette (ABC) transporters (efflux pumps P glycoprotein 1, also known as ABCB1; and ABC subfamily member 2 (ABCG2)) have the ability to nonspecifically scavenge toxic substances; and cells with high levels of ABC transporters are more resistant to cytotoxic chemotherapy. It was found that in primary cell lines derived from neuroblastoma patients, ACD of $ABCG2^{hi}ABCA3^{hi}$ tumor cells generated subpopulations that were $ABCG2^{hi}ABCA3^{hi}$ and stem cell-like, as well as subpopulations that were more differentiated and $ABCG2^{low}ABCA3^{low}$, suggesting that drug pumps were specifically inherited asymmetrically to a subset of daughter cells to maintain the $ABCG2^{hi}ABCA3^{hi}$ cell population that was highly drug-resistant (Hirschmann-Jax et al., 2004). Moreover, a study in neuroblastoma found that CSCs enhanced the therapeutic resistance of daughter cells by asymmetrically co-enriching EGFR and nerve growth factor receptor (p75NTR) in one of the two progeny cells; both receptors in activated state prevent cells from differentiation and enhance the self-renewal capacity of daughter cells (Hitomi et al., 2021).

ACD can also generate and maintain stem cell-like populations with temporary self-renewal capability. Granit et al. discovered a link between asymmetric cell division and the generation of progenitor-like triple negative breast cancer (TNBC) cells. By staining a group of basal-like breast tumors for the basal cell cytokeratin K14, the luminal cytokeratin K18, and the mesenchymal marker vimentin (VIM), Granit found that there were three prominent subpopulations in the tumor samples of invasive TNBC: $K14^{+}K18^{+}$, $K18^{+}$ and $K18^{+}VIM^{+}$, where the $K14^{+}K18^{+}$ subpopulation was luminal progenitor-like and highly tumorigenic. Importantly, they found that progenitor-like $K14^{+}K18^{+}$ cells and luminal-like $K14^{-}K18^{+}$ were able to convert to each other through undergoing ACD. Adjusting the proportion of progenitor-like $K14^{+}$ cells in TNBC tumors by modulating modes of cell division is an important strategy for promoting drug resistance and progression of TNBC (Granit et al., 2018; Ragoussis, 2018). As more and more evidence implicates the contribution of progenitor cells to the progression of tumor malignancy, it is particularly important to identify progenitor-like cells within tumors and to explore the molecular mechanisms underlying ACD that gives rise to progenitor-like tumor cells.

DISCUSSION

Since the discovery of ACD and the conserved pathways associated with it, much effort has been devoted to studying how ACD plays a role in various biological processes. In the past 10 years, there has been tremendous progress made in our understanding of the connection between ACD and cancer. The development of high-throughput sequencing, lineage tracing, and other technologies has opened up a new chapter in the study of the intercellular heterogeneity of tumors (Hajirasouliha et al., 2014; Marjanovic et al., 2020); and ACD as an important source of tumor heterogeneity has attracted

unprecedented attention. People have begun to explore the biological significance of ACD-related mechanisms in generating heterogeneity. To date, researchers have discovered specific mechanisms of ACD under pathological conditions that are used by tumor cells to generate populations with different properties to enrich the intratumoral heterogeneity (Armah, 2010; Pei and Wechsler-Reya, 2010; Granit et al., 2018; Ragoussis, 2018; Hitomi et al., 2021). These findings may provide new ideas for the search of novel therapeutic targets for the treatment of cancer.

Although we have gained some insights into the relationship between ACD and cancer progression, many questions remain to be answered. First of all, the search for new cell types, cellular components, and molecules that can participate in ACD is still on-going. In recent years, asymmetry at the epigenetic level has attracted much attention, as it may provide new insights into tumor heterogeneity at the epigenetic level (Wooten et al., 2020; Zion et al., 2020; French and Pauklin, 2021; Zion and Chen, 2021). Accumulating evidence indicates that progenitor cells may be the origin of certain types of tumors; however, our knowledge of ACD in these cell types is very limited. As for the regulation of ACD, we lack understanding of the difference in regulatory mechanisms of ACD under physiological and pathological conditions at the molecular level. In particular, regulatory pathways that are specifically activated under pathological conditions deserve to be further explored. In addition, a potential link between tumor metastasis and ACD remains to be elucidated, as a few studies have implicated the association between epithelial-mesenchymal transition (EMT) and ACD. A study in non-small cell lung cancer (NSCLC) cells has shown that signaling activities of the aPKC polarity pathway, a core pathway of ACD, is sufficient to prevent EMT (Gunaratne et al., 2013). In turn, in mouse mammary glands, EMT was found to induce ACD for the enrichment and maintenance of the pool of mammary stem cells (Wu et al., 2019). These findings suggest that ACD and EMT may cooperate to facilitate metastasis of tumor cells.

The heterogeneous population of tumor cells generated by ACD is one of the many factors leading to drug resistance and recurrence of tumors. Gaining more knowledge on how ACD plays a role in cancer development will be of great significance for the understanding of tumor malignancy and the search for potential therapeutic strategies. Inducing ACD of CSCs as a differentiation strategy was initially applied to the treatment of patients with simple acute promyelocytic leukemia (APL) in clinic and achieved some success; and this strategy has been tested for the treatment of solid tumors in recent years (de Thé, 2018). A major advantage of this treatment is its low toxicity compared to traditional chemotherapy and radiotherapy, as it does not kill cells directly. However, the potential risks of these therapeutic strategies are poorly understood. Therefore, thoroughly dissecting the mechanistic details of ACD among different cancer types is essential for further development of therapeutic strategies targeting ACD in cancer.

AUTHOR CONTRIBUTIONS

HL concepts formation. ZL and YZ wrote the manuscript. HZ, JY, and YC editing and revision. All authors contributed to the article and approved the submitted version.

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REVIEW
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Moonlighting at the Poles: Non-Canonical Functions of Centrosomes

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Centrosomes are best known as the microtubule organizing centers (MTOCs) of eukaryotic cells. In addition to their classic role in chromosome segregation, centrosomes play diverse roles unrelated to their MTOC activity during cell proliferation and quiescence. Metazoan centrosomes and their functional doppelgängers from lower eukaryotes, the spindle pole bodies (SPBs), act as important structural platforms that orchestrate signaling events essential for cell cycle progression, cellular responses to DNA damage, sensory reception and cell homeostasis. Here, we provide a critical overview of the unconventional and often overlooked roles of centrosomes/SPBs in the life cycle of eukaryotic cells.

Keywords: centrosomes, spindle pole bodies, MTOCs, Cdc5, PLK1, cell cycle

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1 INTRODUCTION

Ever since the centrosome was first discovered in the late 1800s, intense research efforts have been devoted to understanding its roles and life cycle in eukaryotic organisms. In their classic roles as microtubule-organizing centers (MTOCs), centrosomes and SPBs are classified amongst the most primitive organelles but gained complex ancillary functions throughout evolution (Bornens and Azimzadeh, 2007; Nabais et al., 2020). Increasingly, centrosomes are now recognized as important determinants of cell differentiation, self-renewal and aging processes in multicellular organisms.

Visualized for the very first time through electron microscopy, SPBs were described as "small knobs" found at either ends of a long straight fiber during mitosis (Robinow and Marak, 1966). Subsequent studies uncovered that SPBs and centrosomes are morphologically distinct; SPBs are tri-layer structures closely embedded in the nuclear membrane whereas centrosomes are surrounded by pericentriolar material. However, both function as MTOCs. Interestingly, a third class of eukaryotic organelle, the nucleus-associated bodies (NABs), is typically responsible for MTOC-related functions in amoebozoans (Gräf et al., 2015; Gräf, 2018; Ito and Bettencourt-Dias, 2018).

Beyond MTOC activities, centrosomes/SPBs also promote cell signaling events induced by diverse stimulatory and stress signals. Here, we will review the non-canonical roles of MTOCs in cell homeostasis, with a specific focus on how the structural organization and subcellular position of centrosomes/SPBs play a central role in the modulation of cellular processes.

1.1 Function and Structural Organization of Eukaryotic MTOCs: An Overview

1.1.1 Centrosomes as MTOCs

Characterized as a protein-dense scaffolding structure responsible for the nucleation of α- and β-tubulin, centrosomes arrange and anchor microtubules that form the bipolar spindle in mitosis

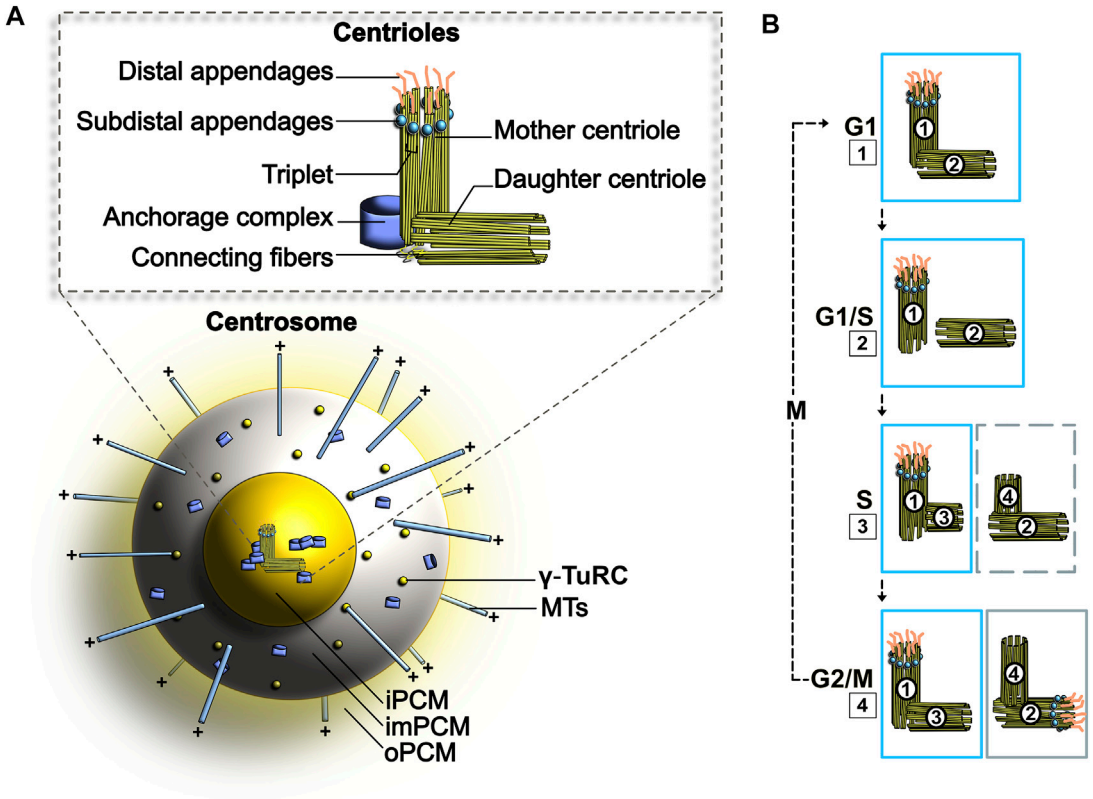

FIGURE 1 | (A) Schematic representation of the centrosome. PCM, Pericentriolar material; iPCM, Inner PCM; imPCM, Intermediate PCM; oPCM, Outer PCM **(B)** Centrosome duplication cycle. The duplication of centrosomes is termed semi-conservative, as each older centriole will generate a new centriole. **1–2**. At the G1/S transition, the two centrioles separate. **3**. In S phase, PCM forms around each parting centriole. **4.** Daughter centrioles expand orthogonally and reach opposite poles. See text for more details.

(reviewed in Wu and Akhmanova, 2017; Gomes Pereira et al., 2021) (**Figure 1**). The main microtubule nucleator is the γ-tubulin ring complex (γ-TuRC), a highly conserved complex responsible for the capping of microtubule minus ends (Oakley et al., 1990; Zheng et al., 1995). Formed of several proteins including γ-tubulin and actin (Liu et al., 2020; Wieczorek et al., 2020), this complex is located in the pericentriolar material (PCM) and was shown to rely on pericentriolar proteins such as CDK5RAP2 to attach to centrosomes (Fong et al., 2008). The γ-TuRC complex, operating as an organizational template for the nucleation of microtubules, forms the cytoplasmic microtubule array in interphase as well as the mitotic spindle during mitosis and was shown to regulate nucleation dynamics via conformational changes (Consolati et al., 2020). From interphase to mitosis, the function of centrosomes as MTOCs is highly dynamic and supports the ongoing division of proliferating cells (Mazia, 1987). Both the size and function of centrosomes as MTOCs may fluctuate according to the state of a given cell, or even its cell type (Decker et al., 2011). To behave in such a dynamic manner, MTOCs rely on centrosomal components and associated proteins that enrich at the centrosomes to stabilize or release microtubule organization and involve a large array of components that can even selectively enrich to one centriole over the other throughout the cell cycle (Andersen et al., 2003; Jakobsen et al., 2011). Combined together, all these factors allow for a personalized MTOC function specifically catered to cell conditions at a given time to accurately support cell cycle progression through microtubule nucleation.

Aside from its classic function as MTOC, the centrosome also plays crucial roles in cell polarity, shape and migration. When Van Beneden first discovered the centriole in 1883 (Van Beneden, 1883), he hypothesized that the polarity of a cell could be conferred by the orientation of both its nucleus and centrosome (Luxton and Gundersen, 2011). The nuclear-centrosomal (NC) axis exists in the majority of metazoan differentiated cell types, as well as in some unicellular organisms including yeast (Nelson, 2003). The polarity of a cell defined by the orientation of its centrosome is an important feature at the core of many biological processes. Research performed on normal fibroblast to study wound healing reported that both the Golgi apparatus and the centrosome (MTOC) were necessary for directional migration towards the edge of a lesion. Authors speculated that the coordinated orientation of both the MTOC and the Golgi apparatus towards the wound was required to modulate vesicular transport to the edge of the cell, thus leading to the growth of this extremity towards the wound (Kupfer et al., 1982).

Akin to this, the centrosome was also reported to play a crucial role in directional mesenchymal cell migration. In a study published in 2017, Zhang and others used micropatterned one-dimensional adhesive strips to study cell polarity in mesenchymal cells and reported that the centrosome was involved in directional cell migration. Specifically, the centrosome was proposed to dynamically localize at the rear of mesenchymal cells to organize the microtubule network and distribute signals related to protrusive activity as a way to establish tail formation during directional migration (Zhang and Wang, 2017).

Asymmetric cell division, a process equally reliant on cell polarity for its occurrence, can also depend on the orientation of centrosomes to effectively reach completion (as reviewed in Chen and Yamashita, 2021). Asymmetric cell division is a common process routinely observed from yeast to humans. In *S. cerevisiae*, aging determinants are partitioned asymmetrically, resulting in a young daughter bud expanding from an older parental yeast. This process directly impacts the replicative lifespan of both parental and daughter cells, which represents the finite number of divisions a cell can undertake before reaching senescence (Longo et al., 2012). Spindle orientation and other factors established by the cell polarity machinery can guide this asymmetric process, which results in the transfer of new components such as mitochondria, endoplasmic reticulum (ER), vacuoles and rejuvenating factors to the daughter cell whilst a number of older components remain in the parental cell (Higuchi-Sanabria et al., 2014). Moreover, SPBs themselves undergo asymmetric inheritance. The older parental SPB migrates towards the new daughter bud, whilst the daughter SPB remains in the parental yeast cell (see section "MTOC duplication cycle" for more details). The asymmetric SPB segregation was shown to be regulated by the spindle positioning protein Kar9 as well as the SPB component Nud1, via its role in the mitotic exit network (MEN) (Hotz et al., 2012a). Along the same lines, asymmetric division is also a feature broadly reported in stem cells, in which the cell type of resulting cells –one self-renewed stem cell and one differentiating cell –differs. In *Drosophila* male germ lines, adult stem cells (GSCs) were shown to asymmetrically divide by relying on the inheritance pattern of mother and daughter centrosomes through directional orientation of the mitotic spindle (Yamashita et al., 2003). Using specific labeling techniques, Yamashita and others later observed that the mother centrosome preferentially remains affixed to the GSCs, whilst the daughter centrosome migrates to the differentiating cell (Yamashita et al., 2007). The authors hypothesized that a high number of astral microtubules may be responsible for the anchorage of the mother centrosome to the GSC, thereby keeping them in close proximity during asymmetric cell division. In accordance with this, the predetermined anchoring of the mother centrosome was suggested to act as an orientation mechanism for the mitotic spindle as a way to ensure the success of asymmetric stem cell division and highlights the core role that centrosomes can play in asymmetric stem cell division. Yet, the non-random segregation of mother and daughter centrosomes is not always a prerequisite for spindle alignment and subsequent asymmetric division. After each of the asymmetric divisions undergone by the germline lineage of the nematode *C. elegans*, centrosome rotation occurs as a way to re-align the spindle to the anterior-posterior (AP) axis. This rotation requires that one of the centrosomes, called the leading centrosome and chosen randomly, travels near the anterior border of the cell (Hyman and White, 1987; O'Connell, 2000). This example demonstrates that the non-random segregation of centrosomes during asymmetric division is a common occurrence in some species and does not represent an essential feature of spindle alignment for asymmetric cell division in all biological systems.

Another important function for centrosomes as MTOCs can also be observed in neuronal development. A decisive part of neuronal differentiation lies in axon specification, a process through which one of the neurites matures into a functional axon. This is of high importance for the fate of a neuron, as this process permanently defines its polarization and connectivity. In the current literature, the contribution of centrosomes to this specific stage of neuronal development has met some controversy (as reviewed in Meka et al., 2020). Several reports describe a key role for the centrosome in axonal outgrowth and specification (Rivas and Hatten, 1995; Schaar and McConnell, 2005; Tsai and Gleeson, 2005; Higginbotham and Gleeson, 2007; Kuijpers and Hoogenraad, 2011), whilst other studies seem to contradict such statement and rule out a potential requirement for centrosome function throughout this neuronal process (Esch et al., 1999; Andersen and Bi, 2000; Bradke and Dotti, 2000). In cultured hipoccampal neurons, growing axons were reported to organize microtubule arrays in a centrosome-independent way once axon specification is complete. This observation is supported by the fact that during axonal elongation, centrosome ablation was shown to have no effect on axon extension or regeneration and suggests that centrosomal function may be required only in the earlier stages of neuronal development (Stiess et al., 2010). Recent studies also argue for a role for centrosomes as F-actin organization centers in developing cultured neurons (Meka et al., 2019). Disruption of centrosome function was shown to alter the content of somatic F-actin and decreased peripheral F-actin matter in neuronal growth cones, suggesting a key role for the centrosome in F-actin organization (Meka et al., 2019). During neuronal differentiation, centrosomes as MTOCs can have various other functions. The most classical and well-known function of MTOCs in neuron biology is probably cargo transport across dendrites and axons, a function performed in partnership with motor proteins (Kapitein et al., 2010). In mouse and chick neural tube cells, centrosomes were also shown to influence neuronal delamination, a process by which novel neurons detach from the neuroepithelium throughout differentiation and morphogenesis. For delamination, the centrosome has to be retained in the newborn neuron and nucleates a wheel-like microtubule organization that supports apical abscission. In this process, the centrosome is thus of high importance in mediating microtubule activity and is involved in nervous system growth and expansion (Kasioulis et al., 2017). Another interesting function for centrosomes in neuron biology is in neuronal activity. Using fluorescent microscopy, Hu and others reported that microtubules also have the propensity to

invade dendritic protrusions. This observation suggests that MTOCs, through microtubules, may have an implication in the operative exchanges between neurons. An increase in neuronal activity was notably shown to correlate with an increased number of spines occupied by microtubules, as well as with an increased contact time between microtubules and dendritic protrusions (Hu et al., 2008). However, more work is needed to establish the precise function of these microtubules in neuronal plasticity. Taken together, these examples display the various ways in which centrosomes as MTOCs can impact neuronal development and highlight the specialized –and still debated– contribution of this organelle in neuron biology.

1.1.2 Structural Organization of MTOCs

Despite lacking a finite membrane border, the centrosome maintains its unique tri-dimensional shape via centrosome-interacting proteins, 500 of which have been identified to date (Andersen et al., 2003; Gupta et al., 2015; Gheiratmand et al., 2019). Throughout the cell cycle, its size and composition vary, allowing for diverse arrangements in microtubule organization (Devi et al., 2021; Gomes Pereira et al., 2021). Centrosomes contain centrioles, a pair of cylindrical organelles perpendicularly positioned to one another (**Figure 1A**). Surrounding the centrioles is the pericentriolar material (PCM), a fibrous coiled-coil protein platform (Schatten, 2008) formed by the main microtubule nucleator γ-tubulin, γ-turc, actin (Liu et al., 2020; Wieczorek et al., 2020) and pericentrin proteins (Salisbury, 1995; Levy et al., 1996; Lutz et al., 2001; Martinez-Campos et al., 2004; Wu and Akhmanova, 2017). This platform allows for sustained or transient anchoring of specific signaling proteins, such as the Nuclear Mitotic Apparatus Protein (NuMA), a key effector of the mitotic machinery. Similar to NuMA, centriolin was also reported to connect to the centrosomes during specific phases of mitosis to facilitate cell cycle progression and cytokinesis (Gromley et al., 2003). Importantly, the size of the PCM varies according to levels of γ-tubulin recruited to centrosomes in a way that supports the ongoing cell cycle state. Accordingly, the PCM is a smaller and tighter structure during interphase and becomes much larger during mitosis to support spindle formation through γ-tubulin nucleation (Robbins et al., 1968; Khodjakov and Rieder, 1999).

Aside from this core centrosomal structure comprised of centrioles and their surrounding PCM, other accessory structures including centriolar appendages and satellites positioned around the PCM further decorate centrosomes and provide this essential organelle with extra key features. The mother and daughter centrioles are different in that additional appendages can only be found on the mother centriole. Distal appendages (DAPs), existing at the distal end of mother centrioles across eukaryotic species except for *C. elegans* and *D. melanogaster* (Azimzadeh, 2014), are required for the docking of the centriole to the membrane and for the process of ciliogenesis (Tanos et al., 2013; Ye et al., 2014). Subdistal appendages (sDAPs) are found in close proximity to DAPs and are also involved in cilia formation and microtubule anchoring. In the literature, the relationship between DAPs and sDAPs remains elusive but recent evidence suggests that DAPs are important for sDAPs functionality and positioning (Chong et al., 2020). Apart from these appendages, centrosomes are also surrounded by centriolar satellites, small particles that congregate around the PCM of centrosomes (Kubo et al., 1999). These satellites are mainly composed of proteins involved in the maintenance of centrosomes, neurogenesis and ciliogenesis (reviewed in Prosser and Pelletier, 2020; Odabasi et al., 2020). Centriolar satellites can also play key roles in the transduction of several other biological cues and vary in form and function throughout the cell cycle and across cell types (Kubo and Tsukita, 2003; reviewed in Tollenaere et al., 2015).

Analogous to centrosomes, SPBs of lower eukaryotes act as key microtubule organizing centers but differ dramatically in their mechanism-of-action and structural features (Jaspersen, 2021). Across yeast species, SPBs are functionally conserved but display key architectural differences. Here, we provide a brief description of both budding yeast and fission yeast SPBs as we compare and contrast their organizational features.

In comparison to the more diffuse centrosomal organization, budding yeast *S. cerevisiae* SPBs are tightly embedded in the nuclear membrane through three highly organized interconnected disk-like structures (see **Figure 2A** for a detailed representation of budding yeast SPB structure) (Robinow and Marak, 1966; Bullitt et al., 1997; Rüthnick and Schiebel, 2018). The outer plaque is responsible for the nucleation of cytoplasmic microtubules, whereas the inner plaque generates nuclear microtubules. The central plaque anchors the SPB into the nuclear membrane and connects to the half-bridge, an important structure for SPB duplication (**Figure 2A**). Two tightly packed and organized disks, called intermediate layer 1 (IL1) and intermediate layer 2 (IL2), act as spacers between the outer plaque and the central plaque. In budding yeast, 17 proteins have been identified as SPB structural components (**Figure 2A**), six of which constitute the core of the spindle pole body: Spc42, Cnm67, Nud1, Spc72, Spc29 and Spc110 (Adams and Kilmartin, 1999; Francis and Davis, 2000; Viswanath et al., 2017). Through reciprocal interactions, these SPB components are integral for creating and maintaining the core SPB structure (Jaspersen and Winey, 2004; Jaspersen, 2021). Most SPB genes are essential for viability and single point mutations in these genes often result in temperature-sensitivity or even lethality.

Fission yeast *S. pombe* SPBs are bipartite structures which, akin to budding yeast SPBs, are implanted in the nuclear membrane. In opposition to budding yeast SPBs, the cytoplasmic domain of fission yeast SPBs represents the bulk of its structure. The architecture of *S. pombe* SPBs also differs from that of budding yeast in that it lacks intermediate spacers and does not contain multiple separate strata except from the outer (cytoplasmic), central and inner (nuclear) layers. Despite these architectural differences, fission yeast SPBs nucleate both cytoplasmic and nuclear microtubules as budding yeast SPBs do and encompass a half bridge required for SPB duplication (Ito and Bettencourt-Dias, 2018). Several components of *S. pombe* SPBs were classified as confirmed or probable homologues of *S. cerevisiae* SPBs, denoting a certain degree of functional and structural conservation in terms of SPB constituents across these species. These include (*S. cerevisiae*/*S. pombe*): Tub4/

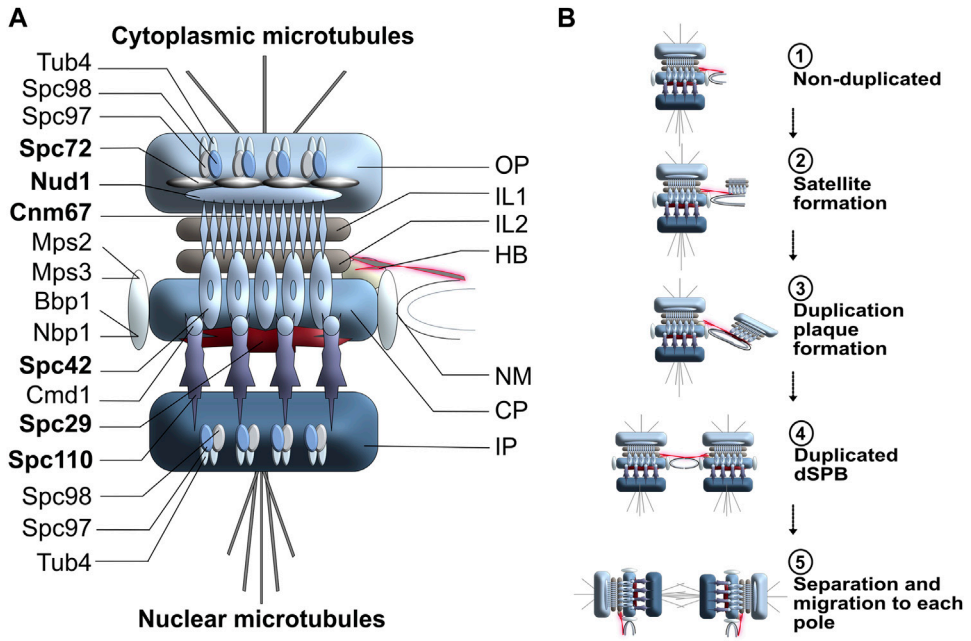

FIGURE 2 | (A) Schematic representation of budding yeast SPB organization and duplication cycle. OP, Outer plaque; IL1, Intermediate layer 1; IL2, Intermediate layer 2; HB, Half-bridge; NM, Nuclear membrane; CP, central plaque; IP; Inner plaque. Core SPB components are highlighted in bold. **(B)** SPB duplication cycle in budding yeast. The duplication process of the SPB is conservative and highly dynamic. Step 1: In early G1, the half-bridge is connected to the SPB central plaque and will act as a scaffold for SPB duplication. Step 2: The half-bridge elongates and the core of the daughter SPB (satellite) is generated on the cytoplasmic face of the half-bridge. Step 3: The duplication plaque, resulting from the elongation and growth of the satellite SPB, matures and mimics the cytoplasmic organization of a mature SPB. Step 4: The half-bridge retracts and fuses to the nuclear membrane. The daughter SPB is assembled and is embedded in the nuclear membrane next to the mother SPB. Step 5: The link between mother and daughter SPBs breaks, leading to the separation of the two organelles.

Gtb1; Spc97/Alp4; Spc98/Alp6; Spc110/Pcp1; Spc72/Mto1; Spc42/Ppc89; Cmd1/Cam1; Cnm67/Sid4; Nud1/Cdc11; Cdc31/Cdc31; Sfi1/Sfi1; Mps3/Sad1; Mps2/Kms2. For more details on *S. pombe* SPB and its structural intricacies, we direct readers towards the study of Bestul et al. (2017).

1.1.3 MTOC Duplication Cycle

Centrosome duplication occurs once per cell cycle and is a semi-conservative process (*i.e.*, the two centrioles present in each cell duplicate to generate two pairs where one template centriole is older than the newly generated copy; **Figure 1B**) (Gomes Pereira et al., 2021). Importantly, the process of centriole assembly occurs throughout three full cell cycles. At the onset of replication, procentrioles (new centrioles) separate and by S^1 phase start their assembly (**Figures 1B**, step 2). Both procentrioles elongate throughout S^1 phase, $G2^1$ phase and M^1 phase and grow perpendicularly from their template side (**Figures 1B**, step 3). At the beginning of M^1 phase, additional PCM is built around each pair of centrioles as they start to separate (**Figures 1B**, step 4). To form the mitotic spindle, procentrioles and their developing centrosome separate in early prophase of M^1. This event, mainly achieved by motor proteins, is supported by push-and-pull forces mediated by the kinesin motor Kif1 and the minus end-directed dynein motor complex. In the literature, the mechanistic intricacies of dynein function in MTOC positioning and separation remained elusive for many years (Holzbaur and Vallee, 1994; Vallee and Sheetz, 1996; Gönczy et al., 1999). Recent studies performed in one-cell *C. elegans* embryos report that different pools of dynein, localized at the cell cortex and on the nuclear surface, can influence centrosome separation. Whilst the pool of dynein located on the nuclear surface moves centrosomes by sliding the centrosome-associated microtubules, the pool of dynein at the cell cortex pulls centrosomes through MT-mediated cortical tugging forces. In this process, dynein was shown to behave as a coupling device that transfers forces produced by polarized actomyosin cortical flows to centrosomes, thereby promoting centrosome separation (De Simone and Gönczy, 2017; Torisawa and Kimura, 2020). Along with this, the plus end-directed kinesin-related motor protein Eg5 creates outward pushing forces by tethering to plus-end antiparallel MTs (Kapitein et al., 2005). Thus, dynein and Eg5 have the ability to create opposite forces that further promotes centrosome separation (Raaijmakers et al., 2012; Agircan et al., 2014). At the end of M^1 phase, procentrioles are separated and individually assemble their PCM. This occurrence, termed centriole disengagement, signifies that the mother and daughter procentrioles are not in close association anymore. Thus, at this stage of centrosome duplication, disengaged procentrioles can be defined as daughter centrioles. From $G1^2$ to S^2 phase of the following cell cycle, daughter centrioles acquire appendages and further increase in length. Upon entry into S^2 phase of the second cell cycle, each newly formed daughter centriole begins

its own cycle of centrosome duplication once more. During this process, the younger mother centriole persistently accumulates additional PCM from S^2 phase to $G2^2$, until its PCM resembles the older mother centriole PCM prior to M^2 phase. In $G2^2$ phase of the second cell cycle, the younger daughter centriole still develops and acquires distal appendages (DAPs) and subdistal appendages (sDAPs). These appendages will evolve and mature until the $G2^3$ phase of the third cell cycle, after which the corresponding round of centriole assembly is complete (Sullenberger et al., 2020).

Centrosome duplication produces two spindle poles that localize perpendicular to the plane of cell division. Achieving this precise orientation is required to support balanced chromosome segregation in mitosis (Kaseda et al., 2012; Silkworth et al., 2012; Nunes et al., 2020). Accordingly, defects in centrosome duplication can have drastic consequences for the cell. If the process of duplication fails and generates extra centrosomes, a resulting scenario may be multipolar mitosis. In multipolar mitosis, chromosomes are segregated to more than two poles during cell division and often leads to gross aneuploidy, chromosome instability (CIN) and clonal evolution (Kwon et al., 2008; Yi et al., 2011; Yang et al., 2012; Telentschak et al., 2015; LoMastro and Holland, 2019). In some cases, clustering mechanisms allow for the formation of a functional bipolar spindle despite the presence of additional centrosomes (Kwon et al., 2008). In other cases, centrosomes may gather at the center of the cell to form a monopolar spindle, a scenario equally threatening to the maintenance of genomic integrity (Chatterjee et al., 2020). Many factors can influence the organization of the mitotic spindle following defective centrosome duplication. Overall, accurate centrosome duplication and partitioning in mitosis is decisive in the maintenance of genome stability and prevention of tumorigenesis.

Like centrosome duplication, SPB duplication is a prerequisite for effective cell division in lower eukaryotes, however, since dynamic exchanges between new and old components occur throughout duplication, SPB duplication cannot be viewed as fully conservative. In budding yeast, the half-bridge elongates in early G1 and remains connected to the central plaque and the IL2 spacer throughout the duplication process (**Figures 2B**, step 1) (Byers and Goetsch, 1974). Once sufficiently elongated, the daughter SPB is built from satellite material (**Figures 2B**, step 2), developing into a duplication structure formed by Cnm67, Nud1 and Spc72 through Spc42-directed self-assembly (Winey et al., 1991; Adams and Kilmartin, 1999) (**Figures 2B**, step 3), after which the half-bridge retracts, allowing for the duplication plaque to embed itself into the membrane. At the end of G1 phase, the parental and daughter SPBs are leveled and connected through a full bridge (**Figures 2B**, step 4), the disassembly of which allows the parental SPB to preferentially migrate into the daughter bud (**Figures 2B**, step 5), and form a bipolar metaphase spindle (Roof et al., 1992; Jaspersen, 2021). Following the initial formation of both spindle poles, additional material is added to each SPB in a dynamic manner, hence why SPBs are considered to be dynamic: their growth process should not be viewed as exclusively conservative (Lengefeld and Barral, 2018). Instead, the continuing SPB maturation increases the ability to maintain functional integrity and has been proposed to be a mechanism for SPB repair (Jaspersen and Winey, 2004).

In fission yeast, the process of SPB duplication differs from that observed in budding yeast. The interphase SPB of *S. pombe* localizes in the cytoplasm, in close proximity to the nuclear envelope (NE), and embeds itself in the nuclear membrane only upon mitotic entry. In the literature, the timing of fission yeast SPB duplication throughout the cell cycle has been controversial for many years. Older studies state that SPB duplication occurs in G2/M (Ding et al., 1997), whereas newer studies suggest that the process instead begins in G1/S phase of the cell cycle (Uzawa et al., 2004). When describing SPB duplication and maturation, Uzawa and others separate the maturation process of *S. pombe* SPB into early and late SPB maturation. Early maturation, reported to occur upon S phase completion, represents growth of the lamellae bodies (laminated structure corresponding to the premature SPB) on the half-bridge, nuclear membrane invagination and gathering of material linking the premature SPB to the nuclear membrane. Akin to what is reported in budding yeast, the early event of SPB duplication giving rise to the lamellae bodies in fission yeast relies on the elongation of the half-bridge. The latter, without which SPB duplication could not take place, is required to support the development of the premature SPB. The newly created laminated structure, still undergoing maturation, remains linked to the mother SPB through an ellipsoid bridge (Ding et al., 1997). Late maturation, shown to take place in M phase of the cell cycle, encompasses the separation of mother and daughter SPBs, NE fenestration for SPB insertion and establishment of the mitotic spindle (Uzawa et al., 2004). While individual steps of SPB duplication differ in some respects across yeast species, the process remains broadly conserved overall.

1.1.4 Centrosomes and SPBs: Same, but Different?

Although centrosomes are significantly larger in size than SPBs (Gräf, 2018), they share several characteristics in duplication modes and main MTOC functions (see **Figure 3** for centrosome/SPB homologs and orthologs). For example, Kendrin and CG-NAP are human orthologs of yeast Spc110 that localize at the PCM (Flory et al., 2000; Takahashi et al., 2002). Likewise, coiled-coil domains required to establish interactions with analogous binding partners are conserved across yeast Nud1 and human centriolin, both of which are important players in cell cycle progression, mitotic exit and cytokinesis (Gromley et al., 2003; Fraschini, 2019) (**Figure 3**). However, microtubules nucleated by the centrosome uniquely enables motility, subcellular trafficking, and anchoring of receptors at the surface of the cell (Bettencourt-Dias, 2013), whereas yeast SPBs remain restricted to roles as MTOCs and docking stations for various signaling events.

2 CENTROSOMES AS SIGNAL TRANSDUCTION ORGANIZING CENTERS

In recent years, an emerging body of evidence support non-canonical roles for centrosomes/SPBs in coordination of signal transduction events (Rincón and Monje-Casas, 2020). Indeed, in response to stimuli and cell cycle cues, kinases with functions unrelated to MTOC activity become transiently enriched at

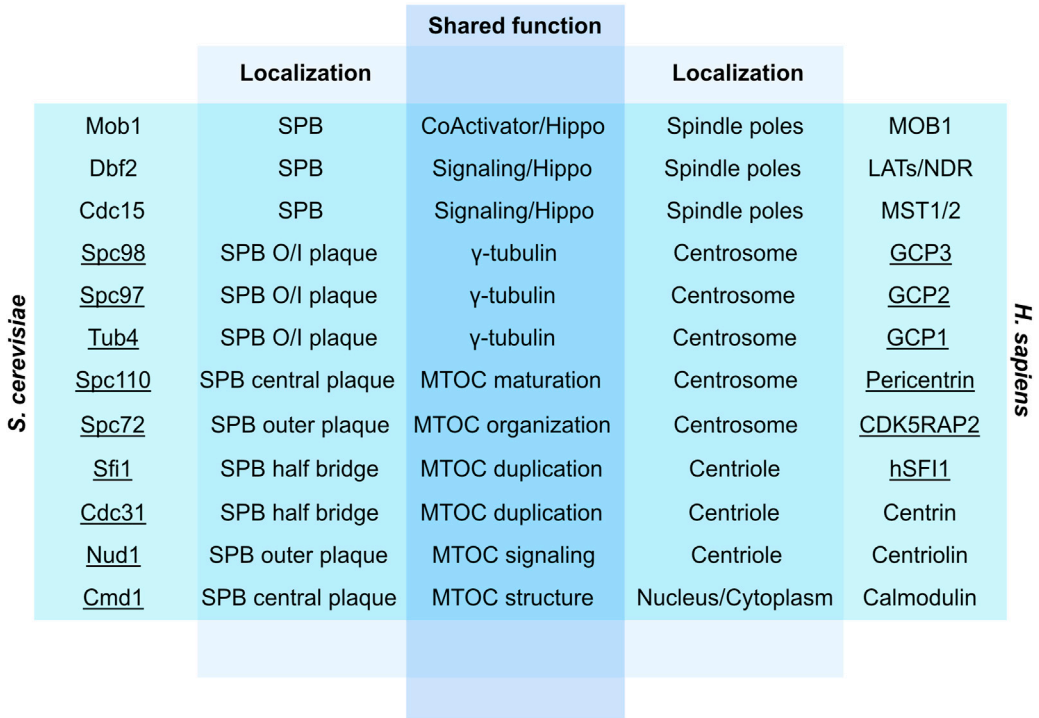

FIGURE 3 | Overview of conserved yeast and human proteins involved in MTOC structure, signaling, duplication and function. Underlined are physical constituents of centrosomes/SPBs. SPB, Spindle pole body; O/I, Outer/Inner; Hippo, Hippo pathway; Pericentrin, Kendrin/CG-NAP (Fraschini, 2019).

centrosomes/SPBs in a manner that is both necessary and sufficient to promote downstream signaling events. Thus, centrosomes/SPBs can modulate kinase activity in a structural capacity as signal transduction organizing centers (STOCs) (Arquint et al., 2014). This function is analogous to that of supramolecular organizing centers involved in the regulation of innate immunity and programed cell death (Kagan et al., 2014), except that centrosome-mediated events occur at much larger and structurally complex scales. In this section, we will explore the surprising relationship between several kinase families and centrosomes/SPBs and how these organelles act as powerful STOCs.

2.1 Centrosomes as STOCs: A Platform to Enable Specialized Functions of Polo-Like Kinases

2.1.1 The Polo-Like Kinase Family

The polo-like kinase (PLK) family, comprised of PLK1-PLK5 in humans, are serine/threonine kinases that regulate fundamental aspects of cell cycle progression (Zitouni et al., 2014; Iliaki et al., 2021). Within this family, polo-like kinase 1 (PLK1) is arguably the most prominent effector of cell cycle events. PLK1 and its functional homologs in budding and fission yeasts (Cdc5 and Plo1, respectively) require phosphorylation by Cdk1/Cdc28/Cdc2 kinases and/or Aurora kinases for full activation *in vivo*. Following this initial activation stage, PLK1 and its yeast counterparts play crucial roles in the regulation of mitotic entry, spindle assembly, chromosome condensation, sister chromatid segregation, cytokinesis, and adaptation to DNA damage (Toczyski et al., 1997; St-Pierre et al., 2009; Ratsima et al., 2011; Zitouni et al., 2014). Importantly, PLK1 function is also essential for centrosome maturation and aberrant PLK1 activity can lead to serious diseases in humans, including cancer (Liu et al., 2017).

All PLKs share a C-terminal polo-box domain (PBD) and a highly conserved multi-domain structure with an N-terminal kinase domain (KD) that harbors a T-loop with an activating phosphorylation site (Rodriguez-Rodriguez et al., 2016; reviewed in; Serrano and D'Amours, 2016). To recognize pre-phosphorylated substrates including CDK1/Cdc28 targets, members of the PLK family use their PBD as a signal amplification module to locate and hyperphosphorylate aforementioned targets. However, the distinctive tripartite architecture of PLK4 PBD differs from the PBDs described across other PLK members and was shown to operate in a phospho-independent manner, making PLK4 PBD an exception on that matter (Slevin et al., 2012). The PBD also behaves as a subcellular targeting domain that allows PLKs to recognize and bind specific structures –such as centrosomes and SPBs– and promote specialized cell cycle functions (Colicino and Hehnly, 2018).

The other members of the PLK family perform distinct but sometimes overlapping functions in cell biology. PLK2, involved in centriole duplication, is dynamically expressed throughout the cell cycle and peaks at the G1/S transition of the cell cycle (Warnke et al., 2004). Given its implication in centriole

biology, PLK2 was reported to endogenously localize at the centrosomes throughout the cell cycle. The expression of PLK2 varies widely across tissues and, given its importance in mammary gland development, was shown to be particularly high in mammary tissues (Villegas et al., 2014). On the other hand, PLK3 is more steadily expressed throughout the cell cycle and its function mostly relates to stress response pathways involving p53 during DNA damage and spindle disruption (Donohue et al., 1995; Xie et al., 2001). PLK4, derived from PLK1 (Carvalho-Santos et al., 2010) and sharing with PLK2 a role in centriole duplication, is characterized as a master regulator of MTOC formation and centrosome amplification (Habedanck et al., 2005). The last member of the PLK family, PLK5, has a slightly different structure than other members of its group in that it completely lacks a kinase domain in humans. Opposite to its other orthologs, the expression of PLK5 was shown to be very low throughout cell division and high in quiescent cells. PLK5 expression is highest in brain tissues and plays a core function in the nervous system, including neuron differentiation (de Cárcer et al., 2011a). For more information regarding the PLK family, its family members and its evolution across species, we direct readers towards reviews covering these topics (Archambault and Glover, 2009; de Cárcer et al., 2011b).

MTOCs are crucial scaffolding structures used by PLKs to reach specific substrates and promote cell division (**Figure 4**). In *S. cerevisiae*, Cdc5 decorates the nuclear surface of duplicating SPBs from late S phase to early anaphase and is also located in the nucleus. In late anaphase, Cdc5 enriches specifically on the cytoplasmic side of the parental SPB segregated to the daughter bud as well as on the bud neck (Botchkarev and Haber, 2017). Once the cell cycle is completed, Cdc5 is degraded by the anaphase-promoting complex (APC) throughout the G1 phase of the next cell cycle (Visintin et al., 2008).

In fission yeast, the polo-related kinase Plo1 shows equally important roles in cell cycle progression and displays high levels of functional overlap with budding yeast Cdc5 and human Plk1 (Lee et al., 2005). Amongst its key roles, Plo1 is required for mitotic entry, formation of the mitotic spindle, establishment of the actin ring prior to cytokinesis as well as septation activation preceding mitotic completion (Ohkura et al., 1995). Similar to Cdc5 and Plk1, Plo1 requires the SPBs as a docking platform and transiently enriches on the structure in a spatiotemporal manner throughout the cell cycle. Similar to the enrichment of Cdc5 at the SPBs, which is low in S-phase but high in G2/M (Simpson-Lavy and Brandeis, 2011), the enrichment of Plo1 on the SPBs is high during mitosis but absent in interphase. Additionally, Plo1 activity at the SPBs is highly reliant on the activity of the kinase Cdc2 (Mulvihill et al., 1999). Upon Cdc2 activation, Plo1 enriches at the SPBs and remains until spindle breakdown whilst keeping steady expression levels throughout the cell cycle (Lee et al., 2005).

The enrichment of Plo1 at the SPBs plays a pivotal role in the commitment to cell division and mitotic progression. The process of mitotic commitment is tightly regulated by M-phase promoting factor (MPF) (Ohi and Gould, 1999), composed of the regulatory subunit cyclin B and the catalytic subunit Cdc2. Following its recruitment to the SPBs in G2 phase (Alfa et al.,

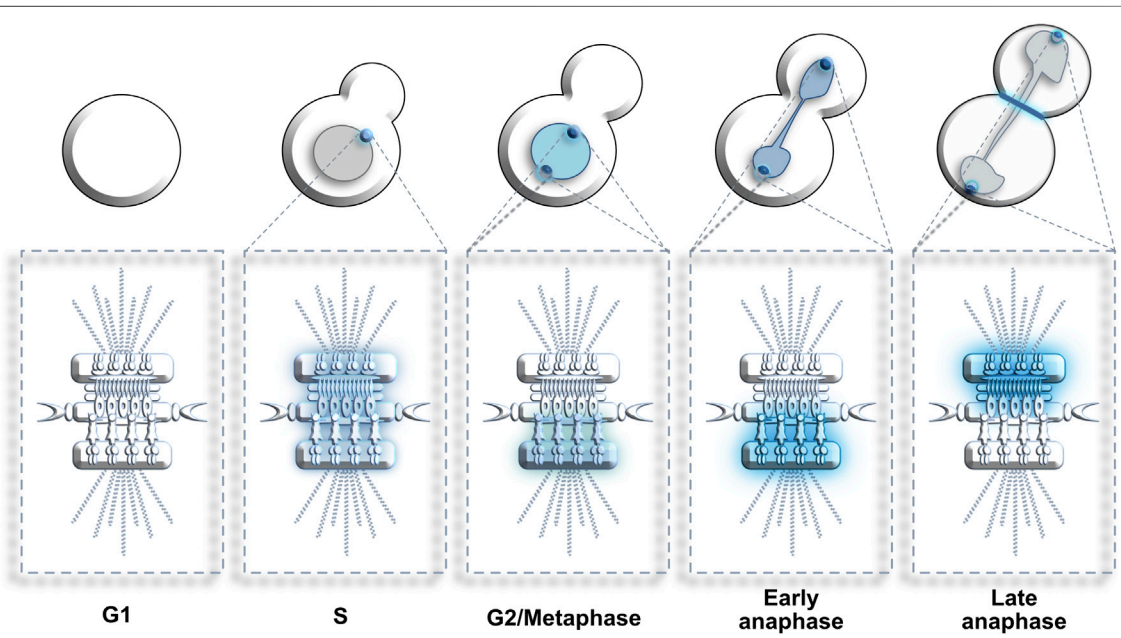

FIGURE 4 | Dynamic localization of Cdc5/Polo kinase at SPBs. G1: Cdc5 is absent from cells. S: Cdc5 enriches at the non-duplicated SPB. G2 to metaphase: Cdc5 decorates the nucleus and the nuclear surface of both SPBs. Early anaphase: Cdc5 concentration at the nuclear surface of both SPBs increases. Late anaphase: Cdc5 relocates from the inner to the outer surface of both SPBs (and bud neck) where it stimulates mitotic exit. Blue color represents enrichment of Cdc5. Color intensity represents Cdc5 concentration levels.

1989; Decottignies et al., 2001; Grallert et al., 2013), MPF activity can promote mitotic entry at any point during the cell cycle. Consequently, its activity must remain strictly restrained to the instant where cell division is timely and suitable. The kinase Wee1, via the phosphorylation of Cdc2, is responsible for such inhibitory effect on MPF activity (Russell and Nurse, 1987). Once all conditions for mitotic progression have been fulfilled, the phosphatase Cdc25 removes the inhibitory phosphorylation on Cdc2 and thus promotes cell division. Once MPF is activated, the complex creates a positive feedback loop that further promotes mitotic commitment through increased Cdc25 activity and Wee1 inhibition. Downstream of this feedback loop instigated by MPF, Plo1 interacts with the SPB component Cut12 in a way that supports entry into mitosis. The NIMA-related kinase Fin1, along with Plo1, was also reported to contribute to this positive feedback loop (Grallert and Hagan, 2002).

Apart from its involvement in mitotic entry, Plo1 is equally important throughout cell division. Plo1 shows two mechanistically distinct activity peaks during mitotic progression: First during prophase, where the formation of the actin ring occurs; second in late mitosis, corresponding to septum formation (Tanaka et al., 2001). Indeed, Plo1 was reported to localize to the medial ring structures as soon as they arise, a subcellular zone that correlates with its key function in the setting of partition sites (Bähler et al., 1998). Akin to Cdc5 in budding yeast, fission yeast Plo1 relies on the APC for its disassociation from the SPBs upon mitotic completion (Mulvihill et al., 1999). Overall, the enrichment of Plo1 at the SPBs is reflective of its implication in the spatial organization of mitotic processes and represents an essential step in the regulation of mitotic entry and cell cycle progression (Lee et al., 2005; Grallert et al., 2013).

In human cells, the Aurora-A kinase, in complex with its co-factor Bora, phosphorylates Plk1 on a conserved residue located in the T-loop of its kinase domain (T210). This G2 phase phosphorylation event uniquely occurs at the centrosomes (Bruinsma et al., 2015). Throughout the cell cycle, Plk1 localization and activity varies greatly. In late G2/early prophase, Plk1 preferentially enriches at the centrosomes to promote mitotic entry and then becomes enriched at the kinetochores to support microtubule-kinetochore connections in prometaphase, with lower Plk1 levels remaining at the centrosomes to instruct spindle assembly.

2.1.2 PLK1 in the DNA Damage Response

The dynamic localization of Cdc5/Plk1 at MTOCs has major implications for signal transduction events during the cellular response to DNA damage. Upon DNA damage, cells initiate a checkpoint response that allows time for DNA repair by preventing the G2/M transition (Sandell and Zakian, 1993; Rhind and Russell, 1998; Cagney et al., 2006; Chao et al., 2017). After successful DNA damage repair, cells resume cycling through a process termed checkpoint recovery (Vaze et al., 2002). However, not all DNA lesions can be safely repaired, and the extent of damage suffered determines the fate of the damaged cells. When DNA damage is too extensive, apoptotic signals lead to programmed cell death thereby preventing the transfer of deleterious genomic errors to daughter cells. When DNA damage is less extensive, cells can resume their cell cycle through checkpoint adaptation (or bypass) despite the presence of "permanent" DNA damage (Sandell and Zakian, 1993; Toczyski et al., 1997; Lee et al., 1998; Vidanes et al., 2010; Ratsima et al., 2011). Consequently, the process of checkpoint adaptation postpones the repair of DNA lesions to subsequent phases of the cell cycle.

The exact signaling pathway responsible for the adaptation response to persistent DNA damage is still not fully understood. In both human and yeast cells, PLK activity is required for adaptation, and Cdc5 enrichment at the SPBs is both necessary and sufficient to promote adaptation to persistent DNA damage in budding yeast cells (Ratsima et al., 2016). These observations suggest that SPBs function as docking platforms for Cdc5 to execute the adaptation response. How this is achieved is unclear, however a possible link connecting PLK, BRCA1 and centrosomes was recently proposed in human cancers (Yoshino et al., 2021). In some cases, aberrant expression of the tumor suppressor gene *BRCA1* in mammary tissues can dysregulate centrosome duplication and generate a higher centriole number *in vivo*. This reported process requires the tethering of BRCA1 to centrosomes via RACK1. This protein also acts as a scaffolding factor that promotes Aurora A and PLK1 interaction in S phase. Previous literature linked RACK1 overexpression to centriole overduplication and involved BRCA1 as a component in this process (Yoshino et al., 2019; Yoshino et al., 2020). This centriole overduplication event was shown to stem from higher levels of phosphorylated PLK1, resulting in kinase hyperactivity at centrosomes. The reported centrosome aberration phenotype in response to PLK1 hyperactivity is intriguingly reminiscent of the supernumerary SPB and polyploidy/multinucleation phenotypes observed in adaptation-defective *cdc5-16* mutants (Ratsima et al., 2011; Ratsima et al., 2016) and in cells overexpressing *CDC5* (Song et al., 2000; Bartholomew et al., 2001). However, more research is needed to assess whether there are cross-species phenotypic similarities between these two cellular processes and how this might be related to the adaptation response to unrepairable DNA damage. Despite the impact of BRCA1 aberrations reported above in centrosome amplification (Yoshino et al., 2019; Yoshino et al., 2020), other studies demonstrated that mutations in BRCA1 can induce a variety of phenotypes that do not always result in amplified centrosomes. To further evaluate the influence of BRCA1 in centrosome biology *in vivo,* Kais and others explored the effect of a subset of mutations in the *BRCA1* locus on centrosome behavior. Remarkably, these mutations induced a range of phenotypes affecting two separate branches of centrosome biology, namely centriole pairing and centrosome number. This result suggests that BRCA1 regulates these two branches of centrosome duplication separately, and nicely underlines the separation-of-function aspect of certain BRCA1 mutations (Kais et al., 2012). Thus, some mutations in BRCA1 can affect functions unrelated to centrosome number and do not always correlate with centrosome amplification in transformed cells.

The process of DNA damage-induced centrosome amplification (DDICA) (Zou et al., 2014) represents another

intriguing link connecting DNA damage responses, PLKs and MTOCs. After treatment with the DNA crosslinker mitomycin C, higher levels of BRCA1 and PLK1 were detected at centrosomes alongside increased centrosome amplification. How DDICA might enhance genomic stability and/or survival remains unclear to this day. On one hand, DDICA may promote the elimination of cells bearing extensive amounts of DNA damage through mitotic catastrophe, whilst contributing to DNA damage repair via local increase of DNA repair factors at the centrosomes (Yoshino et al., 2021). The rationale behind this is that an increased amount of DNA repair factors at the centrosomes stemming from DDICA could constitute an extra source of DNA repair proteins available for relocation from the centrosomes to nuclear sites of DNA damage, consequently supporting nuclear DNA repair as well as DDICA processing. This theory, however, remains to be proven and is a work in progress in the current literature. On the other hand, this process was suggested to be beneficial for cancer cells seeking a proliferative advantage in specific growth environments, as centrosome amplification in p53-deficient cancer cells can encourage chromosome mis-segregation (Yoshino et al., 2021), a key promoter of genomic heterogeneity. The mitotic catastrophe phenotype resulting from DDICA, observed primarily in breast cancer cells, is intriguingly evocative of the phenotype reported in budding yeast with the adaptation-defective *cdc5-16* allele. In response to DNA damage, this mutant fails to enrich at the SPB and gradually fragments its SPB, akin to DDICA (Ratsima et al., 2011). It would be informative for future research to explore the mechanistic similarities between Cdc5-related SPB fragmentation in yeast and PLK1/BRCA1-related DDICA in breast cancer cells.

In damaged cells, the generation of extra centrosomes can also be an outcome of circumstances unrelated to PLK1 or BRCA1 expression. Dodson and others notably reported that centrosome amplification can ensue an extended G2 phase caused by DNA damage checkpoint activation, in which DNA replication is paused but centrosome duplication remains. Interestingly, the small portion of cells able to override this G2/M cell cycle arrest were shown to contain a normal number of centrosomes (Dodson et al., 2004). Other potential causes of centrosome amplification also include cytokinesis failures, as well as cell-cell fusion (reviewed in Godinho and Pellman, 2014). Overall, the relationship between centrosome amplification and DNA damage is an ongoing work in progress in the field of centrosome biology and its intricacies are yet to be fully uncovered.

2.2 Centrosomes as STOCs: PIDDosome Signaling Axis and the Centrosome Surveillance Pathway

Centrosome biogenesis is a process finely coordinated with other cell cycle cues to minimize errors during centriole duplication. In some cases, this control system fails despite its global efficacy and consequently leads to aberrations in centrosome biogenesis. In the literature, centrosome aberrations sometimes are described as a common outcome of neoplastic transformation (LoMastro and Holland, 2019). However, research shows that these aberrations can in fact be at the core of neoplasia, acting as an instigator of cell transformation (Lingle et al., 2002; Pihan et al., 2003; Segat et al., 2010; Lopes et al., 2018; Burigotto et al., 2021). In recent years, a link between centrosomes and the tumor suppressor p53 was unraveled and pointed to a control system for centrosome biogenesis. This control system, termed the PIDDosome signaling axis, acts as a mitotic clock that can detect and react to centrosome aberrations and DNA damage during cell proliferation to monitor and minimize genomic instability (Tinel and Tschopp, 2004; Ando et al., 2012; Ando et al., 2017; Fava et al., 2017; Sladky et al., 2017; Tsabar et al., 2020). The PIDDosome signaling axis is composed of the "cell-death effector caspase-2" (CASP2), the "p53-induced death domain-containing protein 1" (PIDD1) as well as the "CASP2 and RIPK1 domain containing Adaptor with Death Domain" (CRADD). In response to stress signals such as extra centrosomes or genotoxic insults, the local concentration of centrosomal PIDD1 increases and specifically enriches at the mother centriole via ANKRD26, a distal appendage protein (Burigotto et al., 2021). Processing of PIDD1 at the centrosome via auto-cleavage leads to its release in the cytoplasm, where the auto-catalytic and proximity-driven activation of CASP2 occurs (Tinel and Tschopp, 2004). Resulting CASP2 activity stimulates the cleavage of the E3 ubiquitin-ligase MDM2, a negative regulator of p53 stability, ultimately leading to the activation of the tumor suppressor p53 and upregulation of p21, a cell cycle inhibitor (Oliver et al., 2011). To limit cell proliferation, this sequence of events leads to a cell cycle arrest or cell death and thereby supports the maintenance of genomic stability (Evans et al., 2021). The increased local recruitment and resulting enrichment of centrosomal PIDD1 at the distal appendages of the mother centriole is suggested to stem from a cellular surveillance mechanism, in which an abnormally high number of mature centrioles can stimulate the activation of the PIDDosome signaling axis (Fava et al., 2017).

Similarly to the PIDDosome signaling axis in response to centrosome amplification, another pathway termed the centrosome surveillance pathway monitors and reacts to centrosome loss or prolonged mitosis (Lambrus et al., 2016; Meitinger et al., 2016; Lambrus and Holland, 2017). In response to disturbed mitosis, the scaffolding protein 53BP1 acts as a platform to recruit the protein deubiquitinase USP28 as well as p53. The resulting proximity between USP28 and p53 leads to the deubiquitination and subsequent change in p53 activity and p21 upregulation, leading to a proliferation arrest in G1 phase of the cell cycle (Fong et al., 2016; Lambrus et al., 2016; Meitinger et al., 2016). The mechanistic intricacies responsible for the activation of the centrosome surveillance pathway are not fully understood. However, variations in PLK4 expression and activity appear to be linked to centrosome loss and subsequent activation of the centrosome surveillance pathway (Wong et al., 2015). Despite both 53BP1 and USP28 proteins being known binding partners involved in DNA damage response pathways (Zhang et al., 2006; Knobel et al., 2014; Panier and Boulton, 2014; Zimmermann and de Lange, 2014), evidence shows that the activity of the centrosome surveillance signaling pathway is independent from their

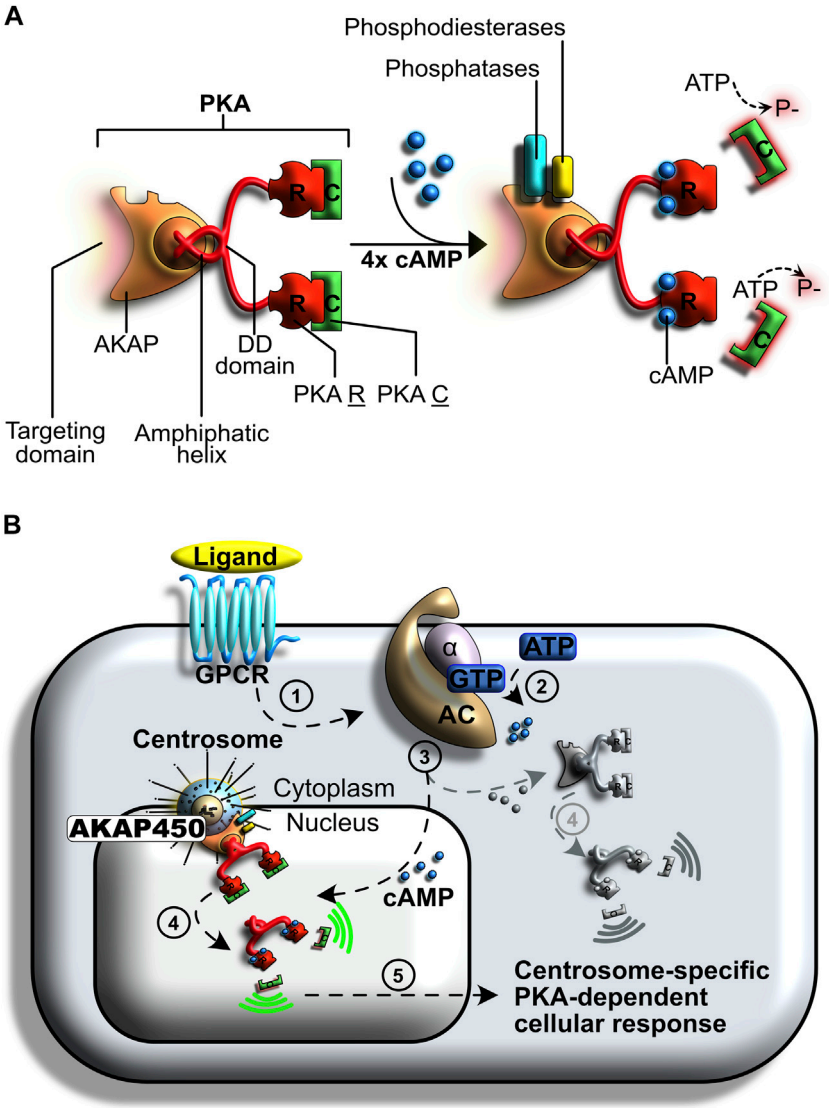

FIGURE 5 | Centrosome-specific regulation of protein kinase A (PKA) signaling. **(A)** PKA is a tetrameric holoenzyme composed of two regulatory subunits and two catalytic subunits. Its activity relies on cyclic AMP (cAMP) cellular levels and is involved in many regulatory processes. **(B)** Regulation of PKA following G protein-coupled receptor (GPCR) activation. A ligand binds to the GPCR (step 1), initiating the signal transduction cascade. This signal induces a GDP to GTP exchange on a heterotrimeric G complex (step 2). The Gα subunit is released and binds to adenylyl cyclase (AC), an event that induces the formation of cyclic adenosine monophosphate (cAMP) from ATP. A subpopulation of PKA anchors at the centrosomes (step 3). The resulting AKAP450 complex increases PKA affinity for cAMP. Centrosomal PKA is selectively activated by cAMP, whilst cytosolic PKA (shown in grey) remains mostly inactive (step 4). A specialized cellular response is induced by the catalytic activation of PKA at centrosomes (step 5).

canonical functions in DNA damage and uncovers a new separate line of defense against the loss of genomic integrity (Lambrus et al., 2016).

2.3 Centrosomes as STOCs: Regulation of Mitotic Entry by cAMP-Dependent Protein Kinase A

Recent work has revealed that PKA activation is regulated differentially in distinct subcellular compartments, and that localized activation sites –known as signaling islands– are key in determining the profile of substrates modified by this kinase (reviewed in Omar and Scott, 2020). PKA localization and its activation kinetics at centrosomes are controlled by kinase-anchoring proteins (AKAPs). Specifically, AKAP450-controlled autophosphorylation of the PKA regulatory subunit lowers the cAMP threshold required for PKA activation at centrosomes (**Figures 5A,B**) (Taylor et al., 1990; Di Benedetto et al., 2008; Taylor et al., 2008; Terrin et al., 2012). In parallel, cAMP-specific phosphodiesterase (PDE4D3) maintains a low cAMP

concentration in the vicinity of this organelle. Combined, these two mechanisms allow for a restricted centrosomal PKA pool to maintain activity when cytosolic PKA is mostly inactive, and thereby promote cell cycle progression without inadvertently inducing gene transcription, signal transduction, or other undesired events.

At the onset of cell division, mitogenic signals trigger an increase in cAMP levels in the entire cell, including the centrosome (Vandame et al., 2014). The increase in centrosomal cAMP is believed to be partly induced by MAPK-mediated inhibition of PDE4D3, which allows the concentration of cAMP to increase (Terrin et al., 2012). However, an exogenous increase in global cellular cAMP levels is not sufficient to induce PKA-mediated cell cycle progression to promote mitosis. Instead, an increase in centrosomal cAMP levels is required; when AKAP450 is artificially relocated away from centrosomes, lack of PKA impairs mitosis and leads to a block in G1 (Gillingham and Munro, 2000; Keryer et al., 2003). Conversely, an artificial increase of centrosomal cAMP levels induces a buildup of prophase cells (Terrin et al., 2012; Vandame et al., 2014).

Together, these studies unraveled that selective activation of centrosomal PKA is pivotal for inducing the cAMP-dependent pathway during mitosis. In this setting, centrosomes act as supramolecular docking platforms in which conditions for PKA activation differ significantly from those that prevail elsewhere in the cell.

2.4 Centrosomes as STOCs: Regulation of Cell Proliferation Decisions by NIMA-Related Kinases

NIMA-related protein kinases are serine/threonine kinases involved in multiple MTOC-related processes. In metazoans, these processes include centrosome separation, during which centrosomes migrate to opposite poles of the cell, spindle assembly, and MTOC-independent regulation of mitotic checkpoints (Nigg, 2001; O'Connell et al., 2003; Moniz et al., 2011; Fry et al., 2012). In humans, seven NIMA-related kinases (Neks) have been identified, whereas lower eukaryotes typically encode a single family member.

The Nek2 isoform in humans is enriched at the centrosomes. Although Nek2 associates with centrosomes in all stages of mitosis, independently of microtubules, its activity is highest in S and G2 phases (Fry et al., 2012). Nek2 is required for centrosome integrity, as evidenced by dramatic phenotypes caused by loss or gain of function mutations. Loss of function mutations were reported to impair centrosome disjunction, a process through which the proteinaceous linker keeping the mother and daughter centrioles in close proximity normally disappears (Hinchcliffe and Sluder, 2001; Fu et al., 2015), and to elicit the formation of monopolar mitotic spindles (Faragher and Fry, 2003; O'Regan et al., 2007). On the other hand, gain of function mutations were reported to induce premature centrosome splitting where a single centrosome would separate into two distinct foci, gradual centrosome loss, and dispersal of centrosomal material (Fry et al., 1998; Fang and Zhang, 2016). Beyond its MTOC-dependent role, Nek2 promotes chromatin condensation in mouse meiotic spermatocytes (Di Agostino et al., 2004; Fry et al., 2012) and cytokinesis in *Drosophila* (Prigent et al., 2005). In fission yeast, the unique Nek2 homolog Fin1 likewise contributes to key cellular processes ranging from mitotic commitment (see section *"Centrosomes as STOCs: Polo-like kinases–The Polo-like kinase (PLK) family"* for an overview of mitotic commitment in *S. pombe*) to spindle function, maintenance of nuclear envelope dynamics and regulation of the septum initiation network (SIN) (Krien et al., 2002; Grallert et al., 2004). Certain phenotypes observed across species upon gain or loss of function mutations in NIMA-related kinases share common themes. Fin1 overproduction in *S. pombe* was notably reported to create spindle formation defects, reminiscent of the centrosome splitting phenotype associated with Nek2 gain of function mutations in humans (Fry et al., 1998; Krien et al., 2002). Despite the lower amount of functional overlap observed in this class of protein kinases in comparison to others (such as PLKs) across species, NIMA-related kinases still share several functional features from yeast to humans and represent an important class of proteins with vital functions in cell biology.

2.4.1 Nek2-Mediated Signaling in the Wnt/Wingless Pathway

Nek2 is known to phosphorylate ß-catenin, a multifunctional Wnt-pathway effector implicated in a wide array of biological contexts including centrosome-related cellular processes (Kaplan et al., 2004; Bahmanyar et al., 2008; Vora et al., 2020). Throughout mitosis, Nek2-mediated ß-catenin phosphorylation prevents its degradation, a mechanism required to maintain high levels of centrosomal ß-catenin (Mbom et al., 2014) and associated with accurate centrosome disjunction. Nek2 kinase activity at the start of mitosis relies on Plk1 (Mardin et al., 2011), however, ß-catenin enrichment at the centrosomes is independent of its phosphorylation state (Mbom et al., 2014). Outside of the centrosome, the Nek2b isoform forms a complex with T-cell factor (TCF4) to drive ß-catenin-dependent cell proliferation, a mechanism associated with tumor cell invasion and metastasis (Shin et al., 2017; Zhang et al., 2017; Shen et al., 2019).

Nek2 also phosphorylates dishevelled (DVL), a scaffold protein involved in both the canonical and non-canonical branches of the Wnt pathway. In higher organisms, three genes encode DVL isoforms –*DVL1*, *DVL2* and *DVL3*. These isoforms, broadly expressed in mammalian cells, were reported to have partly overlapping functions with high levels of redundancy (Lee et al., 2008). The phosphorylation event mediated by Nek2 on DVL isoforms is essential to promote interactions between DVL and several centrosomal linker proteins, liberating these from the centrosomes and ultimately promoting centrosomal separation. Indeed, lack of DVL impedes the dissolution of centrosomal linkers, resulting in an absence of centrosomal separation (Cervenka et al., 2016). Nek2 can also positively modulate the pool of DVL available at the centrosomes to upregulate the canonical Wnt/β-catenin pathway (Cervenka et al., 2016).

Apart from its implication in centrosome separation, the Wnt signaling pathway was also reported to play a role in cell motility. In response to exosome-transported signaling molecules named

planar cell polarity (PCP) proteins, the Wnt pathway stimulates breast cancer cell (BCC) motility at the cell cortex. For this event to occur, the association of a centrosomal module is required. Specifically, DVL2 isoform was shown to mediate the assembly of this module, composed of the human centrosomal protein CEP192 and PLK4/AURKB, to promote protrusive activity in BCCs. This centrosomal module coordinates the exchange of formin DAAM1 for formin DAAM2 at the cell cortex, resulting in increased cell migration (Luo et al., 2019). This sequence of events may partly explain why aberrant expression of PLK4, AURKB and DAAM2 in breast cancer was shown to correlate with poor prognosis and increased cancer aggressiveness (http://www.cbioportal.org). Interestingly, the function of this centrosomal module was reported to be independent of centrosomes or microtubules and elegantly highlights how contextual Wnt signalling in cancer cells has the power to initiate processes such as cell migration as a means to augment metastatic potential.

In the developing *Drosophila* eye, the relationship between Nek2 and Wnt/Wingless is more direct. In a setting where the anaphase-promoting complex (APC) is inactivated, Nek2 accumulation causes hyperactivation of Wnt signaling and blocks retinal differentiation. Conversely, when Nek2 is degraded by APC, local Wnt signaling is suppressed and retinal differentiation proceeds (Martins et al., 2017). Taken together, these studies highlight how Nek2 operates in partnership with the Wnt pathway throughout the entire cell, including at the centrosomes.

2.5 Centrosomes as STOCs: Regulation of Mitotic Exit and/or Cytokinesis by MEN and SIN Kinases

The mitotic exit network (MEN) is a GTPase signaling cascade that regulates cell cycle progression in budding yeast with similarities to the Hippo signaling pathway in metazoans. MEN drives the onset of mitotic exit in late anaphase and cytokinesis primarily by inhibiting the activity of Cdk1 and reversing phosphorylation sites on Cdk1 substrates. SPBs provide spatio-temporal cues for MEN, and importantly, functions as docking platforms to initiate and amplify signaling events.

Up until anaphase, the GTPase Tem1, the main MEN initiator, is present at SPBs but is kept inactive until Cdc14 phosphatase is released from the nucleolus to create a positive feedback loop that drives the mitotic exit process (as reviewed in Manzano-López and Monje-Casas, 2020). Two spindle position checkpoint (SPOC) components, GTPase-activating proteins (GAP) Bfa1/Bub2, inhibit Tem1. In anaphase, spindle elongation allows the older SPB to progressively migrate from the mother cell into the daughter bud, at which point the Cdc5 kinase, enriched at the SPBs, phosphorylates Bfa1/Bub2 to disinhibit Tem1. Concomitantly with this, migration of the older SPB into the bud places the Lte1 guanine-exchange factor (GEF), located in the bud cortex, where it can access and convert Tem1 to its active GTP-bound form. Subsequently, the Cdc15 kinase and its downstream effector –the Dbf2-Mob1 complex– are recruited to SPBs and activated, allowing transmission of the MEN signal to the nucleolus, where it can activate Cdc14 (Renicke et al., 2017; Campbell et al., 2019).

The release of Cdc14 and its gradual accumulation outside of the nucleolus generates a robust feedback loop that promotes mitotic exit (Barberis et al., 2005; Maekawa et al., 2007). Cdc14 enriches at the SPBs via its interaction with the outer plaque component Spc72, and throughout anaphase, gradually increases on the parental/older SPB as it migrates through the daughter bud (Yoshida et al., 2002). In late telophase, once the daughter SPB is fully generated, Cdc14 accumulates on both SPBs. By acting as a docking platform for Cdc14, SPBs may act as a functionally distinct reservoir of active Cdc14 responsible for promoting effective mitotic exit (Yoshida et al., 2002).

The wealth of knowledge on the MEN and its role in mitotic exit sometimes overshadows its equally important roles in cytokinesis. In budding yeast, establishment of an actomyosin ring and septum formation between the mother and daughter bud at the beginning of anaphase are necessary processes for completing cell division and separate the two newly formed cells. Given the temporal pairing of late mitotic events and cytokinesis, many MEN components are also required for the completion of the cytokinetic process. Amongst them, SPB-bound Tem1 and the Bfa1/Bub2 complex were shown to be crucial for successful cytokinesis (Whalen et al., 2018) and the activity of the SPB-enriched Cdc5 kinase required to complete cytokinesis. In late anaphase, Bfa1 maintains Cdc5 mainly on the cytoplasmic side of the daughter SPB (Park et al., 2003). At the onset of cytokinesis, Cdc5 gradually enriches at the bud neck and promotes cell division through its kinase activity towards a specific subset of substrates. The preferential enrichment of Cdc5 at the outer side of the daughter SPB seemingly facilitates the late mitosis/cytokinesis transition by allowing for the rapid migration of Cdc5 at the bud neck (Botchkarev et al., 2017). Thus, the role played by SPBs as platforms that coordinate MEN signaling has implications beyond the area of mitotic exit, such as the regulation of key events required for the completion of cytokinesis.

The septation initiation network (SIN) in fission yeast, a GTPase signaling cascade akin to the budding yeast MEN, regulates several mitotic processes occurring in the last steps of cell division. These processes include actomyosin ring constriction (CAR), septation and cytokinesis (Feoktistova et al., 2012; Alcaide-Gavilán et al., 2014; Edreira et al., 2020). The first event leading to SIN initiation requires the activation of the Ras-like GTPase Spg1 (septum-promoting GTPase) (Schmidt et al., 1997). In metaphase, both SPBs contain uniform amounts of Spg1. The latter, ensuing its activation, recruits its effector protein kinase Cdc7 at the SPBs. Upon anaphase entry, both Spg1 and Cdc7 become inactivated on the parental SPB whilst Cdc7 concentration increases on the daughter SPB (Sohrmann et al., 1998). The resulting asymmetrical enrichment of Cdc7 on the newer SPB further induces the recruitment of Sid1 and Sid2 protein kinases on the daughter SPB, stimulating SIN activity and contributing to the transduction of septation signals from the SPB to the division site (Guertin et al., 2000). Furthermore, Sid2 was reported to exert a positive effect on SIN activity feedback loop, thus maximizing the signaling cascade to promote septation

(Feoktistova et al., 2012). The polo-like kinase Plo1, involved in several steps of mitotic progression, was also reported to positively impact SIN activity and was hypothesized to operate upstream of the aforementioned signaling cascade (Ohkura et al., 1995; Tanaka et al., 2001). Loss-of-function mutations encompassing certain *SIN* genes were reported to induce the formation of elongated multinucleated cells, resulting from the absence of cell division following several cycles of nuclear division (Nasmyth and Nurse, 1981; Balasubramanian et al., 1998). Conversely, gain-of-function mutations were linked to the establishment of numerous actomyosin contractile rings and septa in cells without divided nuclei, a consequence of mutated inhibitors of SIN (Feoktistova et al., 2012).

The function of the MEN in mitotic exit represents a late evolutionary trait. Since the regulation of mitotic exit was coupled to the mitotic exit (ME)-signaling pathway only during the development of the Saccharomycetaceae family, other yeast species such as *C. albicans* or *S. pombe* thus lack this function of the MEN (Maekawa et al., 2022). Moreover, it is worth noting that the MEN was suggested to function earlier in the cell cycle, such as in metaphase, in other processes unrelated to mitotic exit and cytokinesis. SPBs were notably reported to exploit the MEN as a way to drive age-dependent segregation. The spindle positioning protein Kar9 was shown to impact SPB segregation through preferential asymmetric enrichment to the older SPB in metaphase, a process requiring sustained Kar9 phosphorylation by the MEN kinases Dbf2 and Dbf20 (reviewed in Hotz and Barral, 2014). The SPB component Nud1 was also reported to further support the asymmetric enrichment of Kar9 on the old SPB and demonstrates that the MEN can impact cell cycle progression as early as in metaphase, through the establishment of asymmetric SPB inheritance (Hotz et al., 2012a; Hotz et al., 2012b). Importantly, the contribution of the MEN to early mitotic events was shown to be conserved across several eukaryotic species, including *S. pombe*, and suggests that this specific feature of the MEN is a commonly shared evolutive trait (Hachet and Simanis, 2008; Chiba et al., 2009; Chiyoda et al., 2012; Grallert et al., 2012). Despite the fact that cell cycle progression is a collective function of Hippo-related kinases across many eukaryotic species, exceptions remain. The Hippo-related pathway in ciliates was notably reported to contribute to the regulation of cilia biology as well as to the establishment of cell polarity (Tavares et al., 2012; Soares et al., 2019). However, there is no clear evidence that Hippo-related kinases in ciliates regulate cell cycle progression the way it was reported in other species such as yeast and denotes a certain degree of functional variability in this otherwise conserved pathway.

In comparison to the vast body of knowledge collected on the MEN-SPBs relationship in budding yeast or the SIN-SPBs in fission yeast, the precise contribution of human centrosomes to mitotic exit remains relatively unexplored. The Hippo signaling pathway is an important regulator of cell proliferation and apoptosis in higher eukaryotes. Given its importance in chromosome segregation and cytokinesis, the Hippo pathway is thus considered to play a functionally analogous role to the MEN (Hergovich and Hemmings, 2012). Although no clear Tem1 homolog has been identified in humans so far, Ras has been proposed to play a Tem1-like role in mitotic exit. Other MEN components located at SPBs appear to be conserved in humans, for instance centriolin, a centriole-appendage protein that transiently locates at the centrosomes. Thus, centriolin may play a similar role to that of Nud1 in promoting mitotic exit through its protein-protein interactions involving human MEN components (Pereira and Schiebel, 2001; Gromley et al., 2003). The centrosomes appear to act as a scaffolding structure for a broader range of regulators in humans, thus involving them in a multitude of intertwined pathways and cellular processes (Mayor et al., 1999; Chavali et al., 2014).

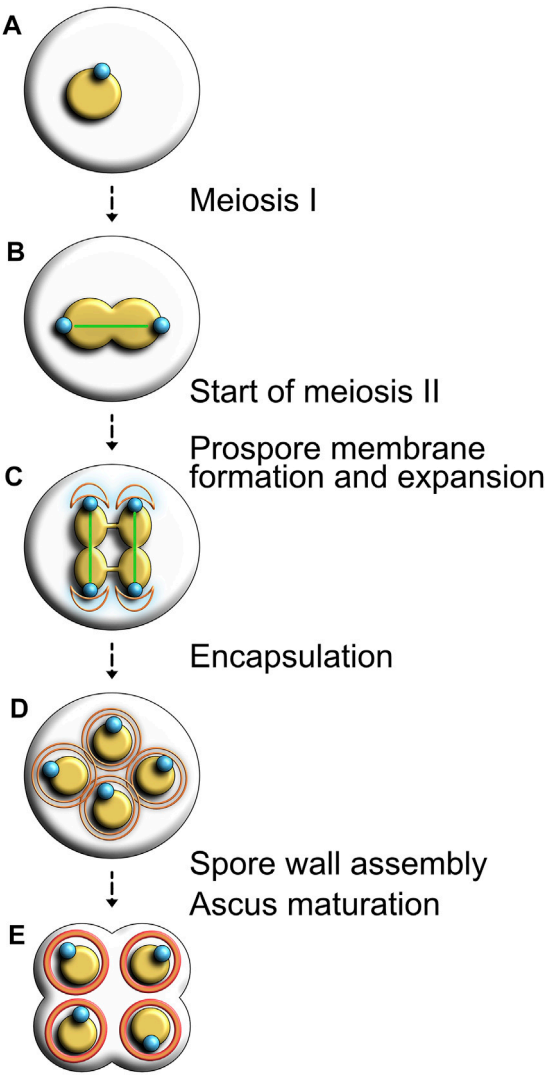

FIGURE 6 | Visual representation of sporulation and ascus formation in budding yeast. **(A)** In response to environmental stressors, diploid yeast cells initiate the sporulation program. **(B)** Completion of meiosis I nuclear division. **(C)** After the second round of chromosome segregation, the prospore membrane (shown in orange) forms and expands around each duplicated SPB (shown in blue). **(D)** The membrane grows and encapsulates each haploid nucleus in the tetrad. **(E)** Spore wall assembly begins and the remnants of the mother cell breaks down.

3 BEYOND MTOC AND STOC ROLES OF CENTROSOMES/SPBS

The studies discussed above describe how SPBs/centrosomes act as essential signaling centers for many biological processes. However, multiple lines of evidence reveal the existence of additional non-canonical roles for centrosomes/SPBs. In this section, we describe how nature and evolution co-opt MTOCs into fulfilling roles that go beyond their typical contribution to cell shape, intra-cellular transport and cell division. These roles require MTOC activity in some cases but utilize microtubules in ways that exceed and/or diverge from their primordial function in eukaryotic cells.

3.1 SPB-Dependent Membrane Formation During Sporulation

When facing environmental stresses or severe nutrient deprivation, organisms ranging from bacteria and protozoa to plants and fungi can undergo sporulation as a way to adapt to environmental changes and increase the likelihood of their survival. Certain eukaryotic species, such as budding yeast, have the capacity to initiate sporulation as a form of specialized meiosis. This meiotic process allows for cells to shuffle and partition their genomic contents into different combinations, thus increasing the likelihood of progeny survival. In yeast, vegetative cells enter into premeiotic S phase. After completion of S phase, replicated DNA is partitioned into four haploid nuclei, which constitute the backbones of the four daughter cells to be created (**Figures 6**, steps A–C). Next, a membrane compartment, called the prospore membrane, matures and surrounds the four newly created daughter nuclei (**Figure 6D**). This step is vital for spore maturation as it gives rise to thick spore walls required for chromatin compaction and protection of cells from harsh environmental conditions (Roeder and Shaw, 1996; Coluccio et al., 2004; Suda et al., 2007; Neiman, 2011). Finally, the remnants of the parental cell collapse around the dormant progeny (the asci) to give rise to four mature haploid cells (**Figure 6E**) (Neiman, 2005).

SPBs support the initial construction of the prospore membrane, but the developmental reprogramming of vegetative cells that leads to sporulation alters their composition and function. During meiosis I, SPB duplication is similar to the process observed during mitotic division, but meiosis II induces multiple changes in SPB constitution that turns this organelle into a focal point for membrane formation. Most of its outer plaque components are replaced with specific proteins required for sporulation. During meiosis II, Mpc54, Spo74 and Spo21/Mpc70, three meiotic plaque components, act as substitutes for Spc72 on the cytoplasmic face of the SPBs. Instead of interacting with microtubules, Mpc54 and Spo21/Mpc70 cooperate with Nud1, Cnm67 and Spc42. The mechanistic process underpinning prospore membrane extension is not well understood, but we know that each prospore membrane surrounds its respective SPB in a semi-circular conformation prior to extension. Each membrane thus captures half of its corresponding nucleus, eventually forming walls englobing the entire nucleus (Neiman, 2011). In their research touching on prospore membrane formation, Knop and Strasser observed that levels of Mpc54 and Mpc70 peaked towards the end of meiosis II and plummeted shortly after, suggesting for a restricted role of these proteins exclusively in the formation of the meiotic plaque. Assembly of the prospore membrane was also shown to rely on Don1, a protein emerging towards the middle stages of meiosis I. Using immuno-electron microscopy, authors reported that Don1 localizes to the prospore wall during meiosis II and was proposed to be a marker for prospore membrane formation (Knop and Strasser, 2000). In a situation where meiotic SPB components are mutated or otherwise deficient, prospore membranes fail to engulf the four nuclei and the sporulation process collapses (Knop and Strasser, 2000), underscoring the essential nature of SPBs for this process.

3.2 MTOC as Linchpins for Cellular Reprogramming of Quiescent Cells

Eukaryotic cells rely heavily on stimuli provided by their immediate surroundings to make cell proliferation decisions. In situations where nutrients become limiting and proliferation is impossible, cells have the ability to initiate stress survival programs that enable them to better face environmental hazards. A cellular state termed quiescence can also be induced when nutrient become scarce or in the presence of specific developmental cues.

Quiescence is a common dormant state in wildlife (Gray et al., 2004; Zhang et al., 2019). Upon entering quiescence, cells temporarily halt their division cycle, thus allowing time for the surrounding environment to replenish its resources (Sagot and Laporte, 2019). This process, routinely observed in unicellular eukaryotes, is also common in multi-cellular organisms including humans, where the quiescent state preserves and maintains embryonic stem cell pools in adult tissues until actively needed for homeostasis or tissue repair (Cheung and Rando, 2013). In yeast, the decision to favor quiescence over other stress coping strategies can be determined by the availability and type of carbon source present in the environment. When ethanol is the predominant carbon source, sporulation and ascospores formation is the main stress coping strategy of budding yeast. Conversely, limited availability of a high-energy fermentable carbon source such as glucose makes quiescence the preferred route to maintain cellular homeostasis and redox equilibrium (Tomova et al., 2019).

Entry into quiescence induces major changes in cellular organization and physiology, including appearance of internal structures such as storage granules and actin bodies (Sagot et al., 2006; Narayanaswamy et al., 2009; Noree et al., 2010; Liu et al., 2012; Laporte et al., 2013; Shah et al., 2013; Sun and Gresham, 2020). The typical Rabl nuclear configuration, in which centromeres are clustered to one side of the nuclear envelope and concomitantly attached to the SPB, is replaced by a simplified nuclear arrangement in quiescent cells (**Figure 7**) (Guacci et al., 1997; Jin et al., 1998; O'Toole et al., 1999; Jin et al., 2000; Bystricky et al., 2004; Laporte et al., 2013). This response is fully reversible because quiescent cells

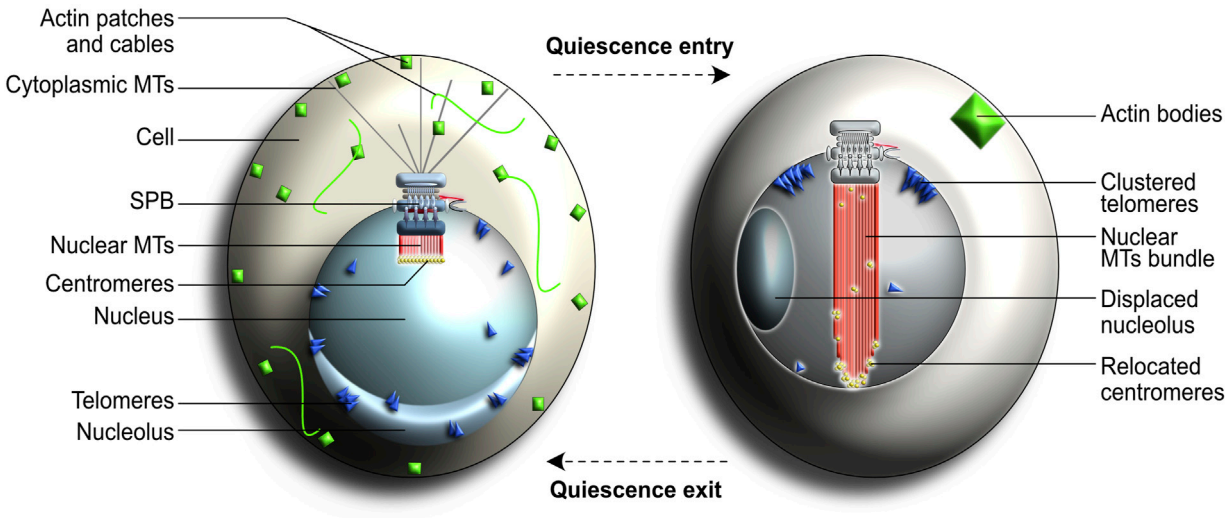

FIGURE 7 | Cellular changes associated with the quiescent state in yeast. These changes include the disappearance of cytoplasmic microtubules (MTs) and formation of a nuclear bundle of MTs (nMTs) that spans the entire nucleus. Centromeres (shown in yellow) normally cluster together at the end of nuclear MTs in interphase cells (left) but get redistributed along the length of the newly formed nMT bundle in quiescent cells (right). Chromosome arms are omitted from this figure to simplify the representation. See text for more details.

typically revert back to the standard Rabl configuration in less than an hour after nutrients are replenished in their immediate environment. This rapid response to environmental cues is highly beneficial for most unicellular organisms and is thought to provide cells with increased competing fitness and enhanced survival chances (Laporte and Sagot, 2014).

An important quiescence hallmark in yeast is the assembly of a long and highly stable array of nuclear microtubules (nMTs) which spans the entire length of the nucleus and consequently displaces the nucleolus (Laporte et al., 2013; Laporte and Sagot, 2014). Chromosomes, which remain tightly attached to the SPB-generated microtubules, become spread along the length of the newly formed nMTs array. Whilst the exact purpose of this nuclear rearrangement during quiescence remains unclear, this selective chromosomal relocation has been proposed to influence gene transcription and mRNA export efficiency (Taddei and Gasser, 2012; Laporte and Sagot, 2014).

SPBs, that form the nMTs array in quiescent cells, are important executioners of the quiescence program. Accordingly, mutations that cause shifts in nMTs array length or stability impede quiescence-related nuclear reorganization and leads to quiescence defects, genomic instability and decreased likelihood of survival (Jin et al., 1998; Gray et al., 2004; Laporte and Sagot, 2014). Likewise, mutations affecting MT nucleation in SPB components, as well as in other organelles or transduction signal pathways involved in quiescence, may drastically reduce cell survival (Gray et al., 2004; Laporte and Sagot, 2014). *XRN1* (also known as *KEM1*) encodes an exonuclease involved in nutrient signaling. Mutated *xrn1* impaired relay of nutritional information to the SPBs, consistent with a possible role for the SPB as a signaling platform during quiescence (Werner-Washburne et al., 1993).

Although a few rare mutant cells survive and are capable of returning to a cycling state upon replenishment of environmental nutrients, the likelihood of survival of their offspring is greatly reduced; a surviving quiescence mutant will confer genomic instability to its progeny, often resulting in cell death (Laporte and Sagot, 2014).

It is unclear if centrosomes play a similar role as SPBs in mammalian cell quiescence. The formation of a nMT array is unlikely to occur in mammalian cells because centrosomes are typically not embedded in the nuclear membrane in higher eukaryotes. However, centrosomes may act as a key docking platform to regulate protein kinase A (PKA) signaling in the early stages of quiescence, as suggested by Gray et al. (2004). Furthermore, the process of quiescence has often been correlated with the formation of a primary cilium in mammals (Tucker et al., 1979; Laporte and Sagot, 2014). Given the requirement for cilium resorption in differentiated cells prior to cell division, the presence of a primary cilium in quiescent cells has been proposed to act as an important biological checkpoint. This theory would satisfactorily correlate with a cell's need to assess the state of its external surroundings prior to reverting back to a cycling state (Kim and Tsiokas, 2011 as cited in; Laporte and Sagot, 2014). Further studies will be necessary to define more precisely the contribution of centrosomes to mammalian cell quiescence.

4 CLOSING REMARKS

Centrosomes and SPBs are cellular organelles mainly recognized for their role as microtubule nucleators (MTOCs) crucial for cell shape determination, intra-cellular transport and cell division. While there is little debate that this viewpoint is well justified, the importance of centrosomes/SPBs in other cellular processes must not be overlooked. Indeed, these organelles also act as key players in the transduction of several signalization events and in the implementation of important

differentiation programs. Through their roles as intracellular docking platforms that enhance kinase-substrate interactions, centrosomes/SPBs effectively function as important STOCs. This role is achieved through the regulated formation of supramolecular protein assemblies on the surface of MTOCs. The scale and compositional complexities of these assemblies suggest that STOCs provide a unique regulatory environment for signaling events. Moreover, the dynamic nature of their location/movements during the cell cycle suggest a capacity for decoding and translating spatio-temporal cues into transduction events. Overall, centrosomes/SPBs are indispensable to ensure cellular fitness and mutations in these organelles can lead to severe pathologies, ranging from microcephaly to cancer (Jaiswal and Singh, 2021). Given their versatile influence in cell proliferation and signaling events, future research efforts focused on the MTOC-independent roles of centrosomes could be a fruitful path for discovering therapeutic targets in the treatment of several diseases, including cancer.

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LL-L and DD'A made a substantial and direct contribution to the work.

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DNA damage checkpoint execution and the rules of its disengagement

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Chromosomes are susceptible to damage during their duplication and segregation or when exposed to genotoxic stresses. Left uncorrected, these lesions can result in genomic instability, leading to cells' diminished fitness, unbridled proliferation or death. To prevent such fates, checkpoint controls transiently halt cell cycle progression to allow time for the implementation of corrective measures. Prominent among these is the DNA damage checkpoint which operates at G2/M transition to ensure that cells with damaged chromosomes do not enter the mitotic phase. The execution and maintenance of cell cycle arrest are essential aspects of G2/M checkpoint and have been studied in detail. Equally critical is cells' ability to switch-off the checkpoint controls after a successful completion of corrective actions and to recommence cell cycle progression. Interestingly, when corrective measures fail, cells can mount an unusual cellular response, termed adaptation, where they escape checkpoint arrest and resume cell cycle progression with damaged chromosomes at the cost of genome instability or even death. Here, we discuss the DNA damage checkpoint, the mitotic networks it inhibits to prevent segregation of damaged chromosomes and the strategies cells employ to quench the checkpoint controls to override the G2/M arrest.

KEYWORDS

cell division, mitosis, checkpoint, DNA damage, adaptation to chromosome damage

Introduction

G1, S, and G2 phases, collectively known as interphase, account for the major portion of the division cycle. G2 phase, though much shorter than G1 and S phases, is an important period in the life of a dividing cell. It not only marks the completion of S phase, but it is also the gateway to mitosis when a cell "prepares'" for a dramatic upheaval in its internal organization. Chromosome condensation, nuclear membrane breakdown, Golgi fragmentation, mitotic spindle assembly, partitioning of duplicated chromosomes and cellular fission collectively represent intracellular organization in a dynamics flux. Soon, the "storm" passes and the progenitor cell undergoes self-cleavage, giving birth to two daughter cells with intracellular organization returning to its stable, interphase state. For the "preparation for M phase," various networks pertaining to mitosis are primed

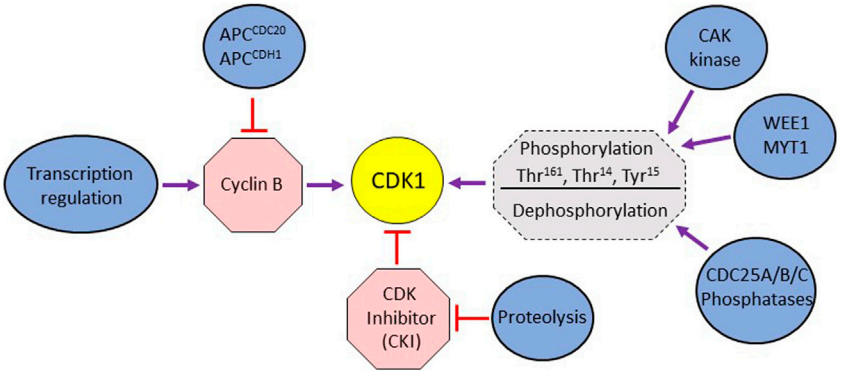

FIGURE 1
(A) Schematic representation of different modes of cellular regulation influencing mitotic activity of CDK1. The activation of CDK1 is directly regulated by cyclin B binding, phosphorylation by WEE1/MYT1 kinases, dephosphorylation by Cdc25 phosphatases and the binding of CDK inhibitors. CDK1's activity is also indirectly affected by transcription regulation impinging on cyclin expression and the proteolytic degradation of cyclins and CDK inhibitors.

(set in a ready-to-go state) in G2 such that all mitotic events are executed in a highly coordinated fashion. Cells that leave G2 phase and enter mitosis prematurely, face uncoordinated passage through M phase, resulting in genomic instability, reduced fitness or death (mitotic catastrophe) (Mc Gee, 2015). The length of G2 varies substantially among different organisms. Unlike vertebrate cells or fission yeast, the G2 phase in the budding yeast *Saccharomyces cerevisiae* is very brief or nonexistent. In *Xenopus laevis*, early embryonic divisions (and in other animal embryos) occur in rapid succession with an apparent omission of G1 and G2 (Siefert et al., 2015). Since mitotic event during these divisions are still executed in a coordinated fashion despite an apparent absence of G2, it suggests that the preparation for mitosis in these division formats begins before the completion of S phase or that there is a nearly complete overlap between the trailing part of S phase and G2.

G2 phase also serves as a "holding room" in the event cells incur DNA damage during S phase. Such damages result in the activation of the "DNA damage checkpoint control," which halts the damaged cells in G2 and prevents them from executing M phase until the damage is fully repaired (Calonge and O'Connell, 2008; Ciccia and Elledge, 2010). In eukaryotes, two major modes of control are used to enact this blockade: by inhibiting CDK1 activation (i.e., onset of M phase) and/or by suppressing chromosome segregation. Once the DNA damage is successfully repaired, cells must disengage the mitotic machinery from the checkpoint control and proceed to mitosis (recovery). Intriguingly, when cells fail to repair the DNA damage, the checkpoint-mitosis disengagement can still occur after a pronged period of arrest and cells enter M phase with damaged chromosomes (adaptation). In this review, we discuss our current understanding of the main mechanisms underlying the activation of DNA damage checkpoint and its deactivation during recovery and adaptation. To set the context, we first briefly describe the mitotic networks, DNA damage checkpoint pathway and the nodes of "contact" between them.

G2-TO-MITOSIS transition and CDK1/CYCLIN B kinase complex

In vertebrate cells, G2/M DNA damage checkpoint halts cell cycle progression predominantly by inhibiting the regulatory network responsible for the entry into mitosis. The master regulator of the G2-to-M transition is the serine/threonine kinase complex CDK1/Cyclin B. The activity of CDK1 is governed primarily by its timely association with cyclin B. While the levels of CDK1 remain stable throughout the cell cycle, the Cyclin B levels fluctuate, reaching their highest during early mitosis and lowest at the end of M phase (Castedo et al., 2002; Sanchez et al., 2003). Cyclin B abundance is regulated at the transcriptional level as well as by proteolysis (Fung and Poon, 2005) (Figure 1). In vertebrates, transcription of Cyclin B is initiated in S phase and peaks in G2 and it is under the control of transcription factors NF-Y, FOXM1 and B-MYB (Lindqvist et al., 2009). Cyclin proteolysis starts during metaphase and continues throughout G1 (Bastians et al., 1999). The proteolytic degradation of Cyclin B is essential for cells' exit from M phase and is mediated by the E3 ubiquitin ligase APC (anaphase promoting complex) (van Leuken et al., 2008). Since many substrates of CDK1/Cyclin B are nuclear proteins, the regulation of cellular localization of Cyclin B is also important for its association with CDK1. During interphase, Cyclin B is actively exported from the nucleus in the export-protein CRM1 dependent manner (Yang et al., 1998). CDK1 and PLK1 have been shown to phosphorylate Cyclin B at the CRM1 binding site, causing cyclin B's net influx into the nucleus (Gavet and Pines, 2010).

The association of CDK1 and cyclin B is stabilized by phosphorylation of Thr^{161} (in human cells; Thr^{167} and Thr^{169} in *Schizosaccharomyces pombe* and *S cerevisiae*, respectively) in the T-loop of CDK1 by CAK kinase (CDK activating kinase) (Ducommun et al., 1991; Tassan et al., 1994; Dalal et al., 2004) (Figure 1). The activity of stable CDK1/cyclin B is negatively regulated by WEE1 kinase family (WEE1 and MYT1) that phosphorylates CDK1 on Thr^{14} and Tyr^{15} residues in the ATP binding site (Schmidt et al., 2017; Moiseeva et al., 2019) (Figure 1). WEE1 mediated phosphorylation holds CDK1 in an inactive state during G2 phase. At the onset of mitosis, the CDC25 family of phosphatases (CDC25A, B, and C in mammalian cells) reverses this inactivation by dephosphorylation of these residues, thereby activating CDK1/cyclin complex. Though CDC25A is predominantly nuclear, the CDC25B isoform shuttle between nucleus and cytoplasm during G2, (Lindqvist et al., 2005; Calonge and O'Connell, 2008; Moiseeva et al., 2019). The cytoplasmic localization of CDC25C during interphase is dependent on its binding to 14-3-3 protein which requires phosphorylation of CDC25C on S^{216} and S^{287} (Dalal et al., 2004). The nuclear translocation of CDC25C is facilitated by phosphorylation of the S^{191} and S^{198} residues (Toyoshima-Morimoto et al., 2002: Bahassi el et al., 2004). Active CDK1/cyclin B complex stabilizes CDC25A, prevents nuclear export of CDC25B and activates CDC25C (Kousholt et al., 2012). Together, the WEE1 kinase family (WEE1 and MYT1) and CDC25 phosphatase family constitute an ON/OFF switch for the CDK1/cyclin B activity (Figure 1). Active Cdk1/cyclin B also phosphorylates WEE1 and MYT1, causing their inactivation (Deibler and Kirschner, 2010). Thus, CDK1/cyclin B-mediated inactivation of WEE1/MYT1 and stabilization of CDC25 phosphatase family sets up a positive feedback loop that helps to amplify its own activity that peaks ~30 min before prometaphase (Potapova et al., 2011). In mammalian cells, polo-like kinase PLK1 also helps to promote entry into mitosis. CDK1-mediated phosphorylation of WEE1 primes it for further phosphorylation by PLK1, thus aiding its inactivation (Ovejero et al., 2012; Parrilla et al., 2016). PLK1 can also activate the transcription factor FOXM1 involved in the expression of CDC25B (Mukhopadhyay et al., 2017). The activity of CDK1 is also regulated by cyclin-dependent kinase inhibitors (CKIs) under certain cellular contexts (Bunz et al., 1998; Satyanarayana et al., 2008).

While finer details of the G2-M transition may differ, the core aspects of CDK1/Cyclin B regulation by phosphorylation are highly conserved in lower eukaryotes such as yeasts *S. pombe* and *S. cerevisiae*. The onset of mitosis in these yeasts is regulated by cdc2 (cdk1)-cdc13 and Cdk1-Clb complexes, respectively (Li et al., 2009; Enserink and Kolodner, 2010). Interestingly, unlike *S. pombe* and mammalian cells, dephosphorylation of Tyr^{15} (Tyr^{19} in *S. cerevisiae*) is not a rate limiting step in *S. cerevisiae* in that the substitution of Tyr^{19} by alanine or inactivation of Swe1 (wee1 equivalent in *S. cerevisiae*) does not lead to premature onset of mitosis (Amon et al., 1992; Booher et al., 1993: Dalal et al., 2004). It is noteworthy that the critical involvement of Cdk1, wee1, APC and other effectors in the regulation of mitotic events was first discovered in these yeasts.

Sister chromatid cohesion and chromosome segregation

The yeast *S. cerevisiae* has been instrumental in the dissection of the DNA damage checkpoint pathway. However, unlike vertebrates, the checkpoint pathway in this organism does not target the events leading up to the onset of mitosis; instead, it inhibits the regulatory network that catalyzes sister chromatid segregation. Following chromosome duplication, the sister chromatids remain associated with each other until they are segregated away during anaphase due to the poleward pull exerted by the mitotic spindle. The cohesion between sister chromatids is mediated by cohesin complex. First reported in *S. cerevisiae*, it is composed of two SMC (Structural Maintenance of Chromosomes) proteins Smc1/Smc3 (Smc1α and Smc3 in human) and a kleisin subunit Scc1 (human Rad21), forming a ring-like arrangement (Makrantoni and Marston, 2018) (Figure 2A). The accessory proteins Scc3, Wpl1, and Pds5 (human SA1/SA2, Wapl, Pds5a/Pds5b) interact with Scc1 and regulate the association of the cohesin complex with the chromatin (Haering et al., 2002; Makrantoni and Marston, 2018). Loading of cohesins occurs prior to replication and is mediated by the Scc2-Scc4 loader. Stabilization of the cohesion complex requires entrapment of both sister chromatids and closing of the cohesin ring (Peters and Nishiyama, 2012). Subsequently, the ring is closed by Eco1 (ESCO1 and ESCO2 in human)-dependent acetylation of Smc3 head on Lys^{112} and Lys^{113}, which prevents the DNA-stimulated ATP hydrolysis and inhibits the opening of the ring (Litwin et al., 2018). The sister chromatid cohesion in yeast is maintained along the entire length of the chromosome until the onset of anaphase (Marston, 2014). In mammalian cells, however, chromosome arm cohesins are removed during prophase ("prophase pathway") in a CDK1-PLK1-dependent manner; only centromeric cohesins (protected by Shugoshin SGO1 and protein phosphatase 2A) persist until the onset of anaphase, giving metaphase chromosome their characteristic X-shape (McGuinness et al., 2005; Haarhuis et al., 2014) (Figure 2B).

At anaphase, sister chromatid cohesion must be dissolved to allow spindle to progressively partition the chromosomes to the opposite poles. In *S. cerevisiae*, the dissolution of cohesion is accomplished by abrupt opening of the cohesin ring by the protease Esp1 (separase or ESPL1 in human) which cleaves the cohesin subunit Scc1, allowing coordinated movement of chromosomes to the opposite poles (Luo and Tong, 2021) (Figure 2C). However, Esp1 remains in an inactive form due its association with securin Pds1 (PTTG in human) until the onset of anaphase (Mei et al., 2001; Han and Poon, 2013). Once all

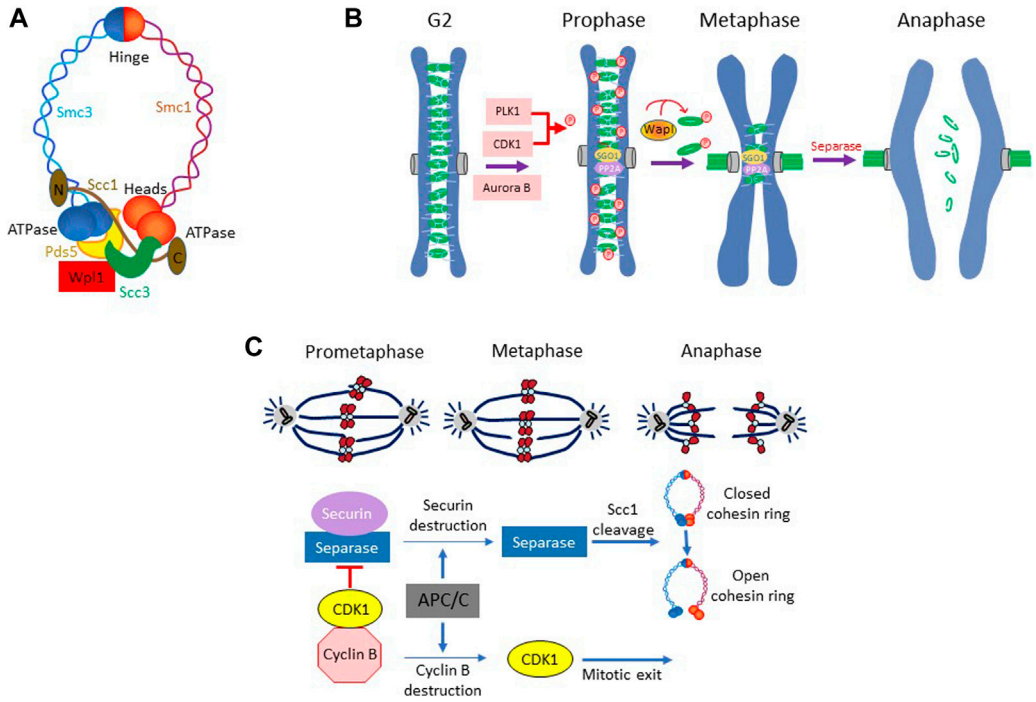

FIGURE 2
(A) The cohesion ring complex and its components in yeast *Saccharomyces cerevisiae*. **(B)** Prophase pathway in metazoan. In metazoans, chromosome arms cohesins are removed during prophase, while centromeric cohesins are protected by SGO1 and PP2A from removal and persists until the onset of anaphase. The removal of the arm-cohesins involves kinase activities of PLK1, Aurora B and CDK1 and the phosphorylation of cohesion subunit SA1 and SA2. In addition, WAPL plays an important role in coordinating cohesion removal during prophase. **(C)** Sister chromatid separation in *S. cerevisiae*. The cohesin complexes in *S cerevisiae* remain in place along the entire length of chromosome until the onset of anaphase. Dissolution of sister chromatid cohesion begins with the proteolytic degradation of securin Pds1 followed by separase mediated cleavage of cohesion subunit Scc1.

chromosomes are appropriately loaded onto the spindle, anaphase is triggered by APC^{Cdc20} mediated proteolytic degradation of securin Pds1 and releasing of separase Esp1 from Pds1-mediated inhibition (Shirayama et al., 1999; Mei et al., 2001) (Figure 2C). Esp1 then cleaves the cohesin subunit Scc1 at the core motif (D/E)-xxR and opens the cohesin ring, allowing spindle-powered poleward movement of sister chromatids (Sullivan et al., 2004; Zhang and Pati, 2017). This regulatory scheme that governs cohesion maintenance and its dissolution is highly conserved between yeast and vertebrates.

G2/M transition under surveillance: DNA damage checkpoint

Double strand breaks (DSBs) are among the most toxic DNA lesions which, if left unrepaired, severely compromise cell survival. Chromosomes are particularly susceptible to damage during replication in S phase. Since segregation of damaged chromosomes during mitosis can greatly exacerbate these damages, it is imperative for cells to halt the cell cycle progression and repair the damage prior to the onset of chromosome segregation. DNA damage response (DDR) is a concerted cellular action plan that integrates 1) the network that detects and processes DNA damage 2) the DNA damage checkpoint that halts cell cycle progression and 3) the system that repairs DSBs *via* homologous recombination (HR) or non-homologous end-joining (NHEJ) (Ciccia and Elledge, 2010; Giglia-Mari et al., 2011; Vitor et al., 2020). Many aspects of the DDR are highly conserved across eukaryotic cells and have been studied in detail in both yeast and vertebrate cells (Stracker et al., 2009; Cussiol et al., 2019).

DNA damage sensing, checkpoint execution and G2 arrest in vertebrate cells

In vertebrate cells, the phosphatidylinositol 3-kinase related protein kinases ATM and ATR, MRN complex, 9-1-1 complex and CHK1/CHK2 are the key elements of the checkpoint activation network. DSB are primarily "sensed/detected" by MRN complex (MRX in yeast) composed of Mre11, Rad50 and Nbs1 (yeast Xrs2) proteins (Tisi et al., 2020; Qiu and Huang, 2021). It carries out

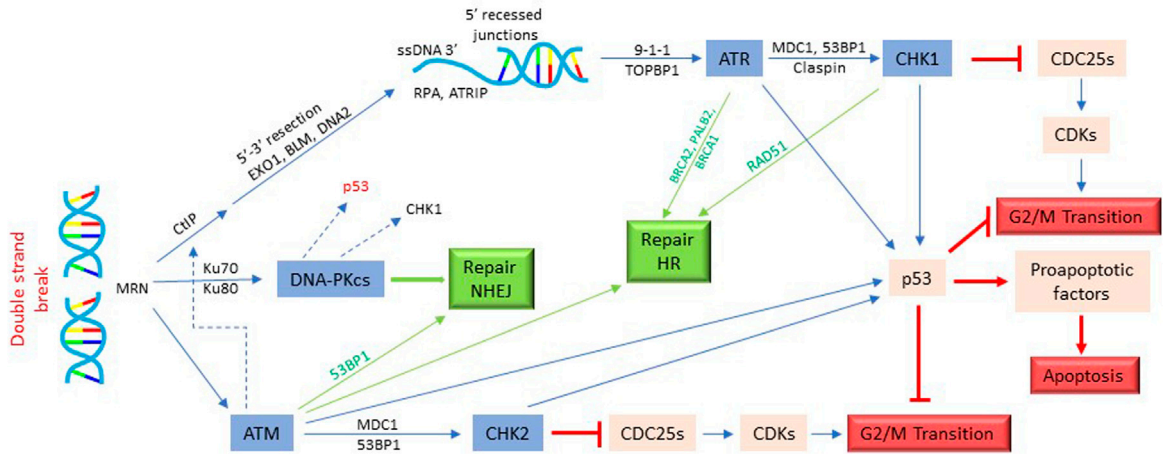

FIGURE 3
Activation of DNA damage checkpoint and cell cycle arrest in mammalian cells. The DSBs in mammalian cells are recognized by MRN complex and processed with the help of CtIP, EXO1, BLM, and DNA2 proteins to generate 3′ ssDNA extension. This results in the recruitment and activation of ATM/ATR kinase and subsequently, the activation of CHK/CHK2 kinases. The activated kinases then inhibit the effectors required for the onset of mitosis, thus causing cells to arrest in G2. In addition, ATR/ATM/CHK1/CHK2 kinases stabilize p53 which help in the imposition of G2 arrest. P53 also activates apoptotic pathway in certain cellular contexts. As in yeast, the activation of checkpoint effectors in mammalian cells also trigger the DNA repair pathways.

initial processing of DSB by generating a short 3′ single strand DNA (ssDNA) overhang (Figure 3). Exo1, a 5′-3′exonuclease, is subsequently recruited for an extensive end-resection to create a long 3′-ssDNA, which is then "coated" by ss-DNA binding factor RPA (Tomimatsu et al., 2017). The RPA-coated ss-DNA is recognized by ATR kinase (Yeast Mec1) *via* its binding partner ATRIP (Ddc2 in yeast). ATR can also be activated by TOPBP1 (yeast Dpb11) recruited at the ssDNA/dsDNA junction (Saldivar et al., 2017). Full activation of ATR also requires recruitment of RAD9-RAD1-HUS1 loader complex (9-1-1 complex: Rad17-Mec3-Ddc1 in yeast) (Delacroix et al., 2007). Another PIKK family kinase ATM (Tel1 in yeast) also contributes to the checkpoint control at G2/M. Transduction of the signal from ATM/ATR to downstream effector kinases involves BRCT-domain containing adaptor proteins 53BP1 and MDC1 and brings these regulators into proximity (Huen and Chen, 2008; Kciuk et al., 2022). CHK1 and CHK2 (yeast Chk1, Rad53) are the main effector kinases activated by ATM/ATR (Figure 3). CHK1 is generally thought to be activated by ATR *via* phosphorylation on S^{317} and S^{345} and CHK2 by ATM *via* phosphorylation on T^{68}. However, given the crosstalk between different axes, these phosphorylation-dependencies may not be strict (Smith et al., 2010). All checkpoint kinases phosphorylate and stabilize transcription factor p53, which is involved in cells' decision to undergo DNA damage-dependent cell cycle arrest, senescence or apoptosis (Oren, 2003; Lavin and Gueven, 2006) (Figure 3).

Once the checkpoint is activated, it targets the mitotic regulators to prevent entry into mitosis. Cdc25C, the phosphatase that plays a critical role in the activation of CDK1, is phosphorylated by ATR, ATM and CHK1 on S^{345} or S^{317} (Liu et al., 2020) (Figure 3). The activation of DNA damage checkpoint also causes phosphorylation of CDC25C on S^{287}, creating a 14-3-3 binding site and preventing it from activating CDK1 (Gardino and Yaffe, 2011). CHK1 and CHK2 kinases phosphorylate CDC25C on Ser^{216}, causing its proteolytic degradation (Hirao et al., 2000; Gottifredi et al., 2001; Liu et al., 2020). CHK1 also promotes degradation of CDC25A by phosphorylation on Ser^{76} (Jin et al., 2008). In addition, checkpoint activation promotes stabilization of CDK1-inhibiting kinase WEE1 by phosphorylation on Ser^{549} and Ser^{287} residues (Lee et al., 2001). Thus, in mammalian cells, DNA damage checkpoint predominantly targets the members of the CDK1-activation network to prevent entry into mitosis. Inhibition of non-CDK1 kinases such as PLK1 and Aurora A also appear to be important in augmenting DNA damage induced G2 arrest (Smits et al., 2000; Peng, 2013; Joukov and De Nicolo, 2018).

DNA damage sensing, checkpoint execution and mitotic arrest in *S. cerevisiae*

Like in vertebrate cells, the DNA damage sensing and its initial processing in *S. cerevisiae* is accomplished by the MRX complex (Figure 4). MRX first attracts Tel/ATM to a unresected DSB. Subsequent localization of Mec1 (human ATR) requires end-resection (Lisby et al., 2004; Gobbini et al., 2016). More extensive

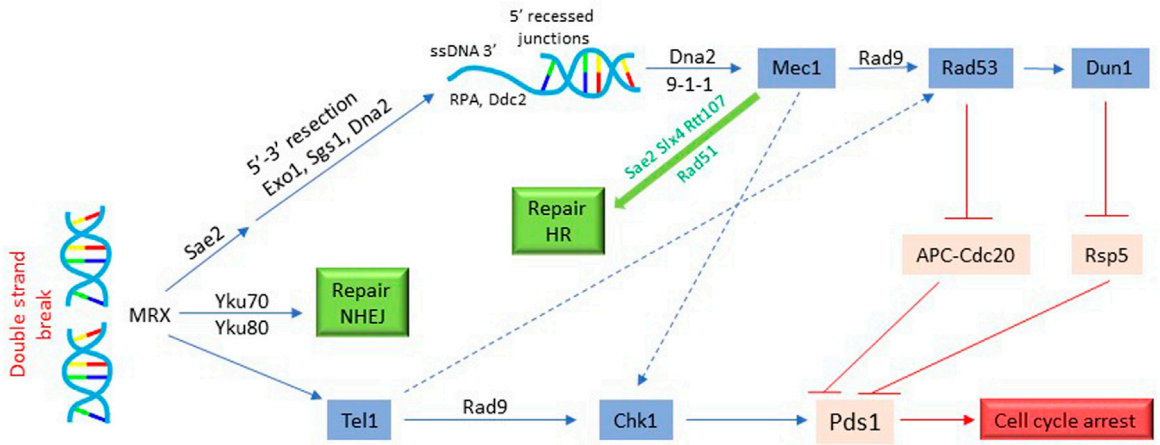

FIGURE 4
Activation of DNA damage checkpoint and cell cycle arrest in yeast. The activation of DNA damage checkpoint in *S. cerevisiae* requires detection of DSB by MRX complex and its processing by Sae2, Exo1, Sgs1, and Dna2 to generate 3′ ssDNA extension. Subsequently, the recruitment and activation of Mec1/Tel1 leads to the activation of Chk1, RAD53 and Dun1, resulting in the phosphorylation of securin Pds1 and protection from proteolytic degradation by E3 ubiquitin ligases APC^{Cdc20} and Rsp5. The stabilized Pds1 inhibits the separase Esp1 and prevents sister chromatid separation, leading to cell cycle arrest. In parallel, the checkpoint activation also triggers the DNA repair pathways.

re-sectioning of the DSB occurs *via* two mechanisms: by Exo1 which removes nucleotides individually from DSB end or by Dna2 endonuclease and Sgs1/BLM helicase (Zhu et al., 2008). Tel1 and Sae2, recruited to the DSB by MRX, initiate Exo1 and Dna2 mediated end-resection (Gobbini et al., 2016; Villa et al., 2016). Cdk1 also plays an important role during resection by phosphorylation and activation of CtIP/Sae2 and Dna2 (Chen et al., 2011; Yu et al., 2019). The localization of Mec1 to the processed DSB (i.e. RPA-coated ss-DNA), is facilitated by Ddc2 (human ATRIP). The adaptor protein Rad9 (mammalian 53BP1) is recruited to the DNA lesions by the scaffold protein Dpb11 (human TOPBP1), which also binds to the 9-1-1 clamp loader *via* Mec1-dependent phosphorylation of Ddc1 (Pfander and Diffley, 2011). Activated Mec1 transmits the damage signal downstream by phosphorylating and activating Rad9 (Figure 4). Mec1-dependent phosphorylation of Rad9 is important for its oligomerization to sustain the damage signal and priming of Rad9 as a scaffold for Rad53/CHK2 localization and for subsequent phosphorylation events (Lanz et al., 2019). Once Rad53 is recruited *via* the docking sites on Rad9, the proximity of multiple Rad53 monomers promotes their autophosphorylation and activation. Activated Rad53 modulates DSB processing by phosphorylating and inhibiting Exo1 (Morin et al., 2008). Mec1 also contributes to the activation of Chk1/CHK1 kinase (Figure 4). While Rad53 is an essential gene for both vegetative growth and DDR in *S cerevisiae*, Chk1 is non-essential for vegetative growth. Chk1 deficient cells exhibit partial defect in G2 arrest in response to ionization radiation (Zachos et al., 2003). Dun1 kinase, a Rad53 paralogue, also features in the dynamics of DNA damage checkpoint (Figure 4). Though structurally similar to Rad53, Dun1 contains a single FHA domain unlike Rad53 that harbors two FHA domains. It interacts with Rad53 through FHA domain and is activated by Rad53-mediated phosphorylation (Bashkirov et al., 2003) (Figure 4). Dun1 is required for DNA damaged-induced transcription of the target genes and the phosphorylation of DNA repair protein Rad55 (Bashkirov et al., 2000; Smolka et al., 2007). It activates the DNA damage-dependent transcriptional program for dNTP synthesis by phosphorylation and degradation of Crt1 and Sml1, both inhibitors of DNA synthesis (Zhao and Rothstein, 2002; Sanvisens et al., 2014). Interestingly, Dun1 deficient cells fail to arrest in response to DNA damage despite the presence of the checkpoint activated Rad53, implying a critical role of Dun1 in DNA damage induced G2/M arrest (Yam et al., 2020) (Figure 4). While the overall scheme of DNA damage checkpoint execution is conserved between yeast and vertebrates, there a few notable differences. For instance, unlike vertebrate cells where tumor suppressor p53 plays an important role in G2 arrest and its cellular outcomes, *S. cerevisiae* lacks p53 homologue or its functional equivalent.

Since G2 phase is extremely brief in *S. cerevisiae* and Tyr^{19} phosphorylation of Cdk1 is necessary but not a rate limiting step for entry into mitosis (Amon et al., 1992; Welburn et al., 2007), the DNA damage checkpoint targets the network that regulate sister chromatid separation. As described above, chromosome segregation in yeast involves cohesion complex, securin Pds1, separase Esp1, APC^{Cdc20} and the spindle apparatus. Mec1-activated Chk1 kinase (and possibly Rad53) phosphorylates Pds1, rendering it resistant to APC^{Cdc20} mediated proteolytic

degradation (Cohen-Fix and Koshland, 1997; Wang et al., 2001; Karumbati and Wilson, 2005). There is also some evidence that Rad53 phosphorylates Cdc20, promoting its degradation and thus contributing to mitotic arrest (Wang et al., 2001). This results in the stabilization of Pds1-Esp1 complex, which prevents Esp1-induced cleavage of the cohesin subunit Scc1 and dissolution of chromosome cohesion (Uhlmann et al., 1999). It has been shown that phospho-Pds1 is further protected from E3 ubiquitin ligase Rsp5 by Dun1 (Liang et al., 2013; Yam et al., 2020). These observations clarify Dun1's role in checkpoint-mediated mitotic arrest. In addition to inhibiting sister chromatid separation, DNA damage checkpoint may also inhibit additional pathways to prevent segregation of damaged chromosomes. It has been proposed that checkpoint maintains APC activator Cdh1 in an active state by inhibiting polo-like kinase Cdc5 (PLK1 in human) to prevent untimely elongation of the mitotic spindle (Zhang et al., 2009).

Switching-Off the checkpoint: Recovery from G2/M arrest following DNA repair

The checkpoint controls remain active during the repair process and continue to prevent cells from progressing to mitosis. Once the DNA repair is completed, the checkpoint controls must be switched off to permit cells to resume cell cycle progression. Since DNA damage checkpoint is activated mainly by protein phosphorylation events, it is not surprising that phosphatases play an important role in its reversal.

Recovery from G2/M arrest in vertebrate cells

In mammalian cells, the redundancy within the CDC25 family of phosphatases comes into play during recovery. As CDC25A and Cdc25C are degraded during G2 arrest, the recovery from checkpoint arrest becomes dependent on CDC25B (Bugler et al., 2006; Chen et al., 2021). Another phosphatase WIP1 also increases in abundance several hours after the induction of damage and accumulates at DSBs (Burdova et al., 2019). WIP1 dephosphorylates and deactivates several checkpoint effectors such as ATM, CHK1 and CHK2 (Goloudina et al., 2016). PP1 and PP2A phosphatases also play an important role in deactivation of the checkpoint in that they dephosphorylate γH2A and inactivate ATM-Chk2 axis (Chowdhury et al., 2005; Campos and Clemente-Blanco, 2020). Mitotic regulators such as PLK1 and Aurora A, and proteolytic degradation also play a significant part in the recovery from checkpoint arrest. Suppression of PLK1 does not affect mitotic entry during normal cell cycle but it significantly delays recovery from checkpoint arrest (van Vugt et al., 2004). This delay can be alleviated by depletion of WEE1 suggesting that WEE1 may be the downstream target of PLK1 during checkpoint recovery (van Vugt and Medema, 2005; Kim, 2022). PLK1 also mediates degradation of Claspin leading to disabling of CHK1 (Mailand et al., 2006; Mamely et al., 2006). CHK2 and 53BP1 are also phosphorylated by PLK1 which disrupts their checkpoint function and helps checkpoint recovery (van Vugt et al., 2010; Peng, 2013). Additionally, Aurora A and its cofactor Bora aid cells' recovery from checkpoint arrest. Since the requirement for Aurora A can be overcome by the expression of activated PLK1, it implies that Aurora A's role in checkpoint recovery is through the activation of PLK1 (Macurek et al., 2008). Inactivation of p53 by PLK1 may also help cells in the resumption of cell cycle progression (Chen et al., 2006). The Greatwall kinase MASTL has been shown to play an important role in regulating mitotic entry during recovery in human cells (Wong et al., 2016). Similarly, in *Xenopus* egg extracts, Greatwall, together with polo-like kinase Plx1, promotes recovery from checkpoint arrest (Peng et al., 2011).

Recovery from G2/M arrest in *S. cerevisiae*

As in vertebrate cells, Ser/Thr phosphatases and proteolytic degradation play an important role in the recovery from G2/M arrest in *S. cerevisiae* (Figure 5A). Since Rad53 is a critical effector in the DNA damage checkpoint, its dephosphorylation has been studied in some detail. PP2A and PP2C classes of phosphatases feature prominently in this context. PP2A Phosphatase Pph3 (and its cofactor Psy2) has been reported to dephosphorylate activated Rad53 and γH2A (O'Neill et al., 2007; Chowdhury et al., 2008; Sun et al., 2011). PP2C phosphatases Ptc2 and Ptc3 (homologues of mammalian WIP1) also dephosphorylate Rad53 after HO endonuclease induced DSB (Guillemain et al., 2007). Ptc2, through its phosphorylation by CK2 on Thr^{376}, dephosphorylates the activated Rad53. There is some evidence that the target specificities of Ptc2 and Ptc3 may not completely overlap (Heideker et al., 2007; Gardino and Yaffe, 2011). The action of these phosphatases may remove some but not all phospho-residues from multiply phosphorylated Rad53, suggesting that other recovery specific processes play important roles in full deactivation of the checkpoint. In yeast, checkpoint attenuation begins during repair of the resected DNA. The repair complex Slx4-Rtt107 loads onto the damage sites, displacing the adaptor protein Rad9 (Cussiol et al., 2015). A similar observation has been reported for Sae2 where it competes with Rad9 for binding to the damage site (Yu et al., 2018). This prevents Rad9 from amplifying the damage signal by Rad9 *via* checkpoint kinases. It is speculated that Srs2 might also be involved in removing checkpoint proteins from the damage site during recovery (Dhingra et al., 2021) In addition, proteolytic degradation of Ddc2, the interacting partner of Mec1, may contribute to the dialing down of the checkpoint signaling during recovery. Ddc2 undergoes Mec1-dependent and

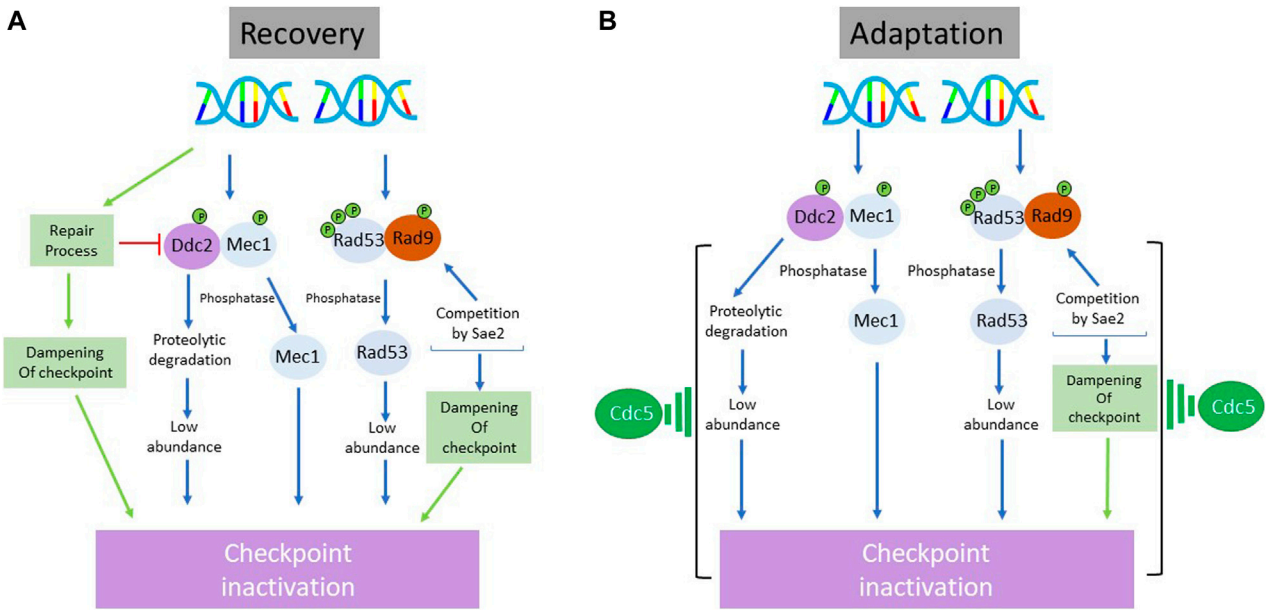

FIGURE 5
Schematic representation of the processes involved in the inactivation of DNA damage checkpoint during recovery **(A)** and adaptation **(B)** in *S. cerevisiae*. While there is a substantial overlap between the regulatory branches utilized to override the checkpoint-imposed arrest during recovery and adaptation, there are two major distinctions: 1) the involvement of repair machinery in the dampening of checkpoint response during recovery but not during adaptation 2) a prominent role of polo-like kinase Cdc5 in checkpoint inactivation during adaptation but not during recovery. Although Cdc5 is firmly implicated in the execution of adaptive response, the molecular details of its exact role are unclear.

-independent phosphorylation upon DNA damage and its abundance decreases as cells recover from checkpoint arrest (Paciotti et al., 2000; Memisoglu et al., 2019). A recent report has proposed a positive feedback loop between Ddc2 stability and checkpoint signaling (Memisoglu et al., 2019). Thus, rapid dampening of the checkpoint controls during recovery period requires inputs from multiple effectors.

Adaptation to DNA damage checkpoint induced arrest: An intriguing cellular behavior

Suspension of cell cycle progression in G2 upon DNA damage and recovery from the arrest following repair of the DNA lesions ensures cells' survival, fitness, and genomic stability. In the event cells are unable to repair DNA damage, they first arrest in G2/M for a long period but then turn-off the checkpoint controls and proceed to mitosis with damaged chromosomes. Mitotic spindle mediated segregation of damaged chromosomes harms the chromosomes further, thus increasing genomic instability or even causing cell death. This cellular behavior, termed adaptation, is intriguing since cells (particularly unicellular organisms such as yeast) derive no obvious advantage from what may appear to be a "self-destructive action." A teleological viewpoint is that adaptation allows cells to escape G2 arrest to attempt DNA repair in the subsequent division cycles i.e., "live to fight another day."

A brief note on the discovery of adaptation

In 1993, Sandall and Zakian reported that elimination of telomere from a chromosome in *S. cerevisiae* results in Rad9-mediated arrest in G2/M (Sandell and Zakian, 1993). Interestingly, many of these cells recovered from the arrest without repairing the lesion and underwent a few cell divisions, eventually losing this chromosome. A critical study by Toczyski, Galgoczy and Hartwell extended these observations and showed that cells that have suffered a single DSB undergo checkpoint induced arrest in G2/M; however, if the DSB cannot be repaired, cells eventually overcome the checkpoint arrest and proceed to mitosis while the damage is still present (Pellicioli et al., 2001). Toczyski et al. (1997) termed this phenomenon "adaptation" and defined three basic criteria for this cellular behavior: 1) cells halt cell cycle progression because of DNA damage 2) cells eventually override the cell cycle arrest 3) cells still harbor the DNA damage at the time they resume cell cycle progression. Subsequently, it was reported that checkpoint signaling is turned off

eventually in *Xenopus* extracts with stalled DNA replication (Yoo et al., 2004). Osteosarcoma cells exposed to ionization radiation (IR) have also been shown to override checkpoint arrest and enter M phase before the damage has been fully repaired (Syljuasen et al., 2006). These studies suggest that cells of multicellular organisms also exhibit "adaptive" behavior when faced with extended checkpoint arrest.

The term "adaptation," derived from "ad + aptus" meaning "toward + fit," is a key concept in the context of evolution. It is a process which allows a population of organisms to accumulate advantageous traits and become better suited to its environment (van Vugt and Medema, 2004; Bartek and Lukas, 2007; Leculier and Roux, 2022). With this general concept in view, the term "adaptation" may seem ill-suited for checkpoint deactivation in response to cells' failure to repair the DNA lesions, as there is little apparent benefit to be gained from this action. It can be argued that switching off the prolonged checkpoint signaling is more akin to desensitization observed in many signaling pathways such as pheromone response in yeast or hormonal response in mammalian cells, which involve feedback inhibition after a significant delay (Jiang and Hao, 2021). Given the transient nature of checkpoint-induced arrest under normal circumstances, it is possible that a deactivation mechanism is built into the checkpoint signaling module. Whether recovery (in repair proficient cells) and adaptation (in repair deficient cells) are mechanistically related events is an important issue.

Dynamics of adaptation and its mechanistic underpinnings in vertebrate cells

In cancer therapy, apoptosis (after 4–6 h of treatment) following DNA damage by anticancer agents has been considered the main pathway for the eradication of tumor cells. However, this model has been supported specifically by data from cancers of myeloid and lymphoid origin. A substantial body of work had suggested that the treatment sensitivity of many tumor cells to DNA damage-causing anti-cancer therapies is due to their failure to repair the damage and to sustain normal DNA damage response, especially when apoptosis occurs 24–48 h after the treatment and usually after mitosis (Brown and Wilson, 2003). This indirectly hints to the failure of tumor cells to maintain the checkpoint-induced arrest. Indeed, attenuation of checkpoint signaling despite the presence of persistent damage is the central element of the adaptive response. Cells can accomplish this in multiple ways. In vertebrate cells, Claspin acts as an adaptor for the recruitment and activation of CHK1. In *Xenopus laevis*, activated ATR, while propagating the checkpoint signaling, also phosphorylates Claspin at Thr^{906} (Lupardus and Cimprich, 2004; Yoo et al., 2004). This creates a docking site for Plx1 (human PLK1) and allows Plx1 to phosphorylate Claspin on Ser^{934}. The modified Claspin then dissociates from the damage-site and undergoes SCFβ-TrCP-mediated degradation (Peschiaroli et al., 2006). These events can result in the dampening of Chk1 activation and checkpoint signal (Mailand et al., 2006). The relationship between CHK1 and PLK1 has also been described in human cells (Tang et al., 2006; Adam et al., 2018). In addition, PLK1 phosphorylates and promotes the degradation of WEE1 which inhibits the CDK1/cyclin B complex (van Vugt and Medema, 2005). In mammalian cells, the level of PLK1 directly affects the cells' ability to adapt. It has been reported that PLK1 activity continue to rise in G2 arrested cells. When this level reaches a threshold, the cells enter mitosis despite the remaining damage (Liang et al., 2014; Verma et al., 2019). This implies that in mammalian cells, adaptation, instead of being an active surveillance mechanism, is a temporal event determined by the level of PLK1 activity (Wakida et al., 2017).

Dynamics of adaptation and its mechanistic underpinnings In *S. Cerevisiae*

Adaptation has been extensively studied in *S. cerevisiae*. Repair-deficient yeast cells suffering a single HO endonuclease-induced DSB arrest in G2/M and subsequently, undergo adaptation (Lee et al., 2000; Dotiwala et al., 2013). However, cells with two DSBs remain permanently arrested (Lee et al., 1998). There is evidence to suggest that cells do not respond to the number of DSBs, but to the extent of single stranded DNA produced by processing of the DSBs (Lee et al., 2001). Upon detection of the irreparable DSB, the checkpoint is activated, leading to phosphorylation of Rad53, Chk1, Ddc2, and Cdc20 and cells arrest in G2/M for an extended period (Waterman et al., 2020). Subsequently (~10 h after the introduction of DSB), Rad53 is dephosphorylated, Ddc2 diminishes in abundance and cells harboring the DSB proceed to mitosis (Memisoglu et al., 2019). It is noteworthy that having undergone adaptation once, yeast cells continue to divide without reactivating the checkpoint in the subsequent rounds of division cycles. As repeated divisions cause further accumulation of chromosome aberrations, most of these cells eventually lose viability. IR-exposed human osteosarcoma cells undergoing adaptation also accumulate chromosome aberrations (Kalsbeek and Golsteyn, 2017), implying that checkpoint adaptation response in mammalian cells increases the risk of accumulating genetically abnormal cells that could potentially undergo malignant transformation.

An early genetic screen in yeast to identify genes involved in adaptation to HO-induced single DSB yielded yeast polo-like kinase *CDC5* and *CKB2*, a non-essential subunit of casein kinase II (Toczyski et al., 1997). Subsequent studies identified additional genes that play a role in checkpoint adaptation, namely, phosphatase Ptc2 and Ptc3 (Leroy et al., 2003), telomeric Ku complex subunits Yku70 and Yku80 (Lee et al., 1998), helicase Srs2 and DNA-dependent ATPase Tid1 (Ferrari et al., 2013; Bronstein et al., 2018). Ptc2 and Ptc3 dephosphorylate Rad53 to

terminate checkpoint signaling (Figure 5B). Phosphorylated by Casein kinase II on Thr^{376} located in a TXXD motif, Ptc2 can bind to the FHA1 domain of Rad53 and facilitate dephosphorylation of Rad53. Consistent with this, *ptc2Δ ptc3Δ* double mutant exhibits normal cell cycle kinetics but fail to undergo adaptation (Guillemain et al., 2007). In yku80 mutant, Ku DNA binding complex is disrupted and resection is accelerated; consequently, cells become permanently arrested in G2/M (Clerici et al., 2008). Of all the genes influencing adaptive response, Cdc5 kinase has garnered much attention. DNA damaged cells harboring adaptation-defective *cdc5-ad* allele remain permanently arrested in G2/M, even though Cdc5-ad retains its kinase activity (Toczyski et al., 1997; Rawal et al., 2016). Cdc5 overexpression, on the other hand, accelerates adaptation and partially suppresses other adaptation defective mutants (Donnianni et al., 2010; Vidanes et al., 2010). In Cdc5 overexpressing cells, though the early steps of checkpoint activation such as recruitment of Ddc1/Ddc2 and Mec1 activation remain unaffected (Donnianni et al., 2010), Rad53 hyperphosphorylation is conspicuously reduced (Vidanes et al., 2010). The mechanism by which Cdc5 kinase diminishes the hyperphosphorylation of Rad53 kinase is not clear. Cdc5 can phosphorylate Rad53 and this modification appears to be important for adaptation (Schleker et al., 2010). It is possible that Cdc5-mediated phosphorylation of Rad53 triggers its deactivation. However, it is uncertain if Rad53 dephosphorylation is the main factor in promoting adaptation since two other mutants *cdc5-16* and $cdc5^{T238A}$ remain arrested after Rad53 is dephosphorylated (Ratsima et al., 2016; Rawal et al., 2016). In yeast, the Mec1-Ddc2 complex localizes to the damage-site, triggering the recruitment and activation of Rad53. Turning off the checkpoint during adaptation may also involve these upstream dynamics. There is evidence to suggest that Mec1 is phosphorylated on S^{1964} residue and that the activated Ddc2 is degraded during adaptation (Memisoglu et al., 2019). These events will limit Mec1 localization to the break site and consequently, attenuate the checkpoint signaling cascade (Bandhu et al., 2014).

Cells carrying uncapped telomeres also exhibit checkpoint activation and adaptation. Single strand DNA present at the telomeres is normally capped by CST (Cdc13-Stn1-Ten1) and Ku (yKu70/yKu80) complexes (Westmoreland et al., 2018), that prevent activation of DNA damage signaling. The yeast ts mutant *cdc13-1* is defective in telomere capping, which results in an extensive resection by Exo1 (Langston et al., 2020), leading to the activation of DNA damage-checkpoint signaling and G2/M arrest. Just as in the case of cells harboring HO-induced single DSB, *cdc13-1* cells undergo adaptation involving Cdc5 and CK2 and undergo accelerated adaptation in response to Cdc5 overexpression (Ratsima et al., 2016; Coutelier et al., 2018).

Recovery and adaptation: Same exit different doors?

A release from the arrest-state and resumption of cell cycle progression are the phenotypic outcome of both recovery and adaptation. In both cases, the checkpoint signaling is turned off and mitotic machinery is re-engaged; however, the cellular contexts are very different. While cells recovering from G2 arrest do so after the DNA damage has been repaired, cells undergoing adaptation override arrest when the damage is still present. As discussed above, during the repair-driven recovery, checkpoint signaling is dampened in step with the DNA repair. However, terminating the upstream signals is not sufficient for recovery; the protein modifications of checkpoint effectors present during G2 arrest must also be reversed. In mammalian cells, WIP1, PP1 and PP2A dephosphorylate γH2A and inactivate ATM-Chk2 axis (Ramos et al., 2019). In yeast, phosphorylated Rad53 is a prominent player in the checkpoint signaling and is dephosphorylated by Ptc2/Ptc3 phosphatases during recovery (Leroy et al., 2003). In addition, the regulators of mitosis play an important role in the process of recovery. Although polo-like kinase PLK1 or CDC25B phosphatase are not strictly required for mitotic entry in undamaged cells, both these regulators are important for the recovery from DNA damage-induced G2 arrest (van Vugt et al., 2005; Bansal and Lazo, 2007; Hyun et al., 2014). PLK1 also promotes proteolytic degradation of the CDK1 inhibitory kinase WEE1 (van Vugt et al., 2004).

The adaptive response shares some regulatory aspects with the recovery process. Most prominent in this context is the role of phosphatases: WIP1, PP1, and PP2A in mammalian cells and Ptc2/Ptc3 in yeast (Heideker et al., 2007; Freeman and Monteiro, 2010). It is not clear, at least in yeast, whether Ptc2/Ptc3 phosphatases act constitutively or require adaptation-specific activation. Since the option of dampening down of upstream events by repair complexes (during recovery in yeast) is not available during adaptation, limiting the continued activation of these events by other means is important (Mailand et al., 2006). In mammalian cells, involvement of polo-like kinase is another element shared by both recovery and adaptation response (Liang et al., 2014). PLK1 can phosphorylate and promote the degradation of WEE1 during both responses (Takaki et al., 2008). However, the role of polo-like kinase Cdc5 in adaptation in yeast is somewhat perplexing. That adaptation defective (and repair proficient) *cdc5-ad* mutant can efficiently recover from DNA damage-induced arrest implies that Cdc5 is not required for recovery (Pellicioli et al., 2001; Vidanes et al., 2010). The fact that both *cdc5-ad* and *cdc5Δ* mutants are adaptation deficient and overexpression of Cdc5 accelerates adaptation suggests that Cdc5 is a key rate limiting factor for the adaptative process (Shaltiel et al., 2015; Serrano and D'Amours, 2014). As dephosphorylation of Rad53 is one of the prominent features of

cells undergoing adaptation, it is not clear how Cdc5 causes dephosphorylation of Rad53. It has been reported that Cdc5 does not inhibit the formation of Rad9-Rad53 complex but does prevent hyperphosphorylation of Rad53 (Vidanes et al., 2010). Since Rad53 is proposed to prevent BRCT-SCD domain-specific oligomerization of Rad9 required to maintain checkpoint signaling (Usui et al., 2009), it is possible that this action of Rad53 limits its own activation. As Cdc5 also inhibit Rad53 autophosphorylation *in vivo*, this may result in the enhancement of the negative feedback loop between Rad9 and Rad53 (Usui et al., 2009; Lopez-Mosqueda et al., 2010; Vidanes et al., 2010). As Rad53 has been reported to inhibit Cdc5 in response to DNA damage (Sanchez et al., 1999; Coutelier et al., 2021), this can potentially add an additional regulatory branch. Important as dephosphorylation of Rad53 during adaptation is, it remains unclear if this is the primary event that initiates adaptation. Recently, a change in the abundance of Mec1-associated protein Ddc2 is suggested to be an important event in adaptation in response to a persistent DSB (Memisoglu et al., 2019).

Closing remarks

Halting of cell cycle progression by checkpoint mechanism in response to DNA damage is a critical aspect of survival for a dividing cell. Cells utilize various means to temporarily decouple from the division protocol to execute the repair program. Reengagement of the division machinery after this transient hiatus is equally important and requires disconnection from the checkpoint protocol. The nature of this reengagement and its outcome is dependent on the execution of repair program: successful repair maintains genomic stability, enhances viability (recovery) and results in healthy progeny, whereas failure to repair results in genomic instability and loss of viability (adaptation). Checkpoint needs to be extinguished during both recovery and adaptation. In organisms as distantly related as yeast and human, recovery and adaptation involve some common strategies and regulatory elements to extinguish the checkpoint (Figure 5). However, there are some notable differences. Since the repair process itself acts to diminish the checkpoint signaling, DNA repair and recovery are closely coupled events. In repair-deficient (or inefficient) cells, the repair mediated whittling down of checkpoint signaling is not an option available during adaptive response. Therefore, cells must achieve it by employing other strategies in which Polo-like kinase plays a critical role as described in the previous sections. Importantly, it is unclear when cells 'decide' to give up attempting to repair the DNA damage and to initiate the adaptative process. Based on the available evidence, adaptation does not appear to be an active process. Rather, it may be a timed response in that the checkpoint erosion is naturally coupled to its activation. Unless aided by the DNA repair system, the natural checkpoint inactivation is perhaps a slow process requiring progressive accumulation of some effectors or establishment of some feedback loops. This would explain why adaptive response is so prolonged an event. Nevertheless, as cell cycle reentry in the presence of DNA damage (as in adaptation) is a harmful undertaking resulting in genomic instability, adaptive response may be relevant to cancer progression in multicellular organisms. Upregulation of proteins implicated in recovery/adaptation is reported in many cancers and is correlated to poor treatment outcomes. A deeper understanding of the regulatory interfaces between DNA damage/repair/checkpoint controls/recovery/adaptation would be relevant to cancer prevention and treatment.

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Conflict of interest

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

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Cyclin-dependent kinase 6 (CDK6) as a potent regulator of the ovarian primordial-to-primary follicle transition

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Introduction: Ovarian follicle development requires tight coordination between several factors to initiate folliculogenesis to generate a mature and fertile egg. Studies have shown that cell cycle factors might contribute to follicle development, hover specific knowledge on individual CDKs and follicle activation has not been investigated. Among cell cycle regulators, *CDK6* is a key player through binding to cyclin D resulting DNA synthesis and genome duplication. Interestingly, the *CDK6* gene is differentially expressed in oocytes and granulosa cells from human primordial and primary follicles, which suggest a potential role of *CDK6* in the primordial-to-primary transition. In this study, we investigated the potential regulatory role of *CDK6* in progression of primordial to primary follicle transition using BSJ-03-123 (BSJ), a CDK6-specific degrader.

Methods: In mouse ovarian *in vitro* culture, BSJ reduced the activation of primordial follicles, and reduced follicle development. As a next step, we examined the egg maturation read-out and found that BSJ-treated follicles matured to competent MII eggs with resumption of first meiosis, comparable with the control group.

Results: Noteworthy, it appears that inhibition of *CDK6* did increase number of apotoptic cells, articular in the granulosa cells, but had no impact on ROS level of cultured ovaries compared to control group, indicating that the cells were not stressed. Oocyte quality thus appeared safe.

Discussion: The results of this study indicate that *CDK6* plays a role in the primordial-to-primary transition, suggesting that cell cycle regulation is an essential part of ovarian follicle development.

KEYWORDS

primordial follicle activation, CDK, oocytes, ovary, *in vitro*

Introduction

The cell cycle progression is a vital and highly preserved physiologic process, which controls the genome duplication (Ding et al., 2020). In mammals, a highly stage-managed series of mitotic and meiotic cell cycles is required for folliculogenesis and oogenesis. During gonadal development, oogonia begins prophase of the first meiotic division and proceed through leptotene, zygotene and pachytene stages, followed by arresting in diplotene stage to become oocyte. The oocyte stays in the diplotene stage and is surrounded by a single layer of flattened pre-granulosa cells, forming dormant (primordial) follicles. In a process known as primordial follicle activation, a cohort of primordial follicles is activated into primary follicle stages (Persson et al., 2005), a follicle stage characterized by an oocyte surrounded by a single layer of cubical granulosa cells. Granulosa cells progress dynamic control of mitotic cell cycle process during folliculogenesis. At the end of follicle development, the mature oocyte arrest after the first meiotic division. Both meiotic and mitotic cell cycle processes are developmentally regulated and playing important role during folliculogenesis and oogenesis (Persson et al., 2005). The cell cycle process is governed by several cyclins and cyclin-dependent serine/threonine kinases (CDKs) (Ding et al., 2020).

CDKs include over 20 members and with their sequential activation and phosphorylation, in association with D-type cyclins, regulate the exit from G1 to S phase in cell cycle (Tigan et al., 2016). Among CDKs, CDK6, along with its partner CDK4, are key players in cell cycle progression through binding to cyclin Ds resulting phosphorylation of the retinoblastoma protein (Rbp), and inducing DNA synthesis. One targeted therapy for CDK inhibition is palbociclib, which may slow the growth of advanced stage breast cancers. In line with this, Palbociclib was used to protect chemin-induced human granulosa cells from apoptosis, acting to inhibit the p53/p21 pathway (Li et al., 2019).

The activity of CDK4 and CDK6 is controlled by p16, whereas p27 bind to a broad range of CDK-cyclin complex. Previous reports showed p27 to be a suppressor of ovarian follicle endowment/formation and activation, and an enhancer of ovarian follicle atresia (Jirawatnotai et al., 2003). On the other hand, the early oocyte and follicle growth was coincided with reduction in p16 expression demonstrating the relationship between CDK6 and follicle development (Bayrak and Oktay, 2003). In line with this, the Cyclin dependent kinase inhibitor 1B (CDKN1B) controlled ovarian development in mice by suppressing follicle endowment and activation, and also promoting follicle death (Rajareddy et al., 2007), comparable to p27. CDKN1B controls the activation of cyclin CDK6/4-cyclin D complexes, and thus governs the cell cycle progression at G1 (Nebenfuehr et al., 2020). In a recent study conducted in porcine oocytes, inhibition of CDK2, CDK4, and CDK6 was not influential on cumulus expansion or germinal vesicle breakdown, whereas CDK7 and CDK9 inhibition reduced germinal vesicle breakdown and cumulus expansion (Oqani et al., 2017).

Previous data revealed that the CDK6 gene was expressed in human primordial and primary follicles, where transcriptional levels of *CDK6* showed downregulation in oocytes and upregulation in granulosa cells, from primordial to primary follicle transition in human ovarian tissue (Ernst et al., 2017; Ernst et al., 2018).

In order to determine the possible role of CDK6 in the primordial-to-primary transition, BSJ-03-123 (BSJ), a degrader with proteome-wide selectivity for CDK6, which uniquely enables rapid pharmacological interrogation of CDK6-dependent functions, was used. To address the role of CDK6 in follicle development. BSJ was added to primary ovary organ culture to assess the effect *in vitro*. Interestingly, BSJ inhibited primordial follicle activation in a concentration-dependent manner, aligned with a reduced level of CDK6 protein, suggested the possible role of CDK6 in primordial follicle regulation. Moreover, the ability of the BSJ-treated follicles to mature to MII oocytes was revealed, suggesting BSJ as a future clinical candidate for regulating primordial follicle activation, eg., during chemotherapy, to prevent premature ovarian exhaustion.

Results

CDK6 was present in human primordial and primary follicles

In the present study, we extracted the *CDK6* gene expression from transcriptomic analysis studies applied in isolated human oocytes and granulosa cells from primordial and primary follicles (Ernst et al., 2017; Ernst et al., 2018).

Interestingly, transcriptional level of *CDK6* was downregulated from 6.66 to 1.61 fragments per kilobase of exon model per million reads mapped (FPKM) (mean) in oocytes from primordial to primary follicle transition (Figure 1A). In contrast, *CDK6* expression was upregulated in granulosa cells from 2.37 to 3.54 FPKM from primordial to primary follicle activation (Figure 1A). In summary, as the *CKD6* transcript is reduced in human oocytes from the primordial-to-primary transition, the *CDK6* transcript is slightly upregulated in the granulosa cells. In the same transition, suggesting an active role of CDK6. To interrogate the CDK6 protein expressed, immunofluorescence analysis on human ovarian tissue using a CDK6 antibody was performed. The Immunofluorescence results showed the presence of the

A

Follicle stage	Primordial follicle				Primary follicle			
Gene name	Oocytes		Granulosa cells		Oocytes		Granulosa cells	
	FPKM mean	P value	FPKM mean	P value	FPKM mean	P value	FPKM mean	P value
CDK6	6.66	**0.001**	2.37	0.22	1.61	0.18	3.54	0.12

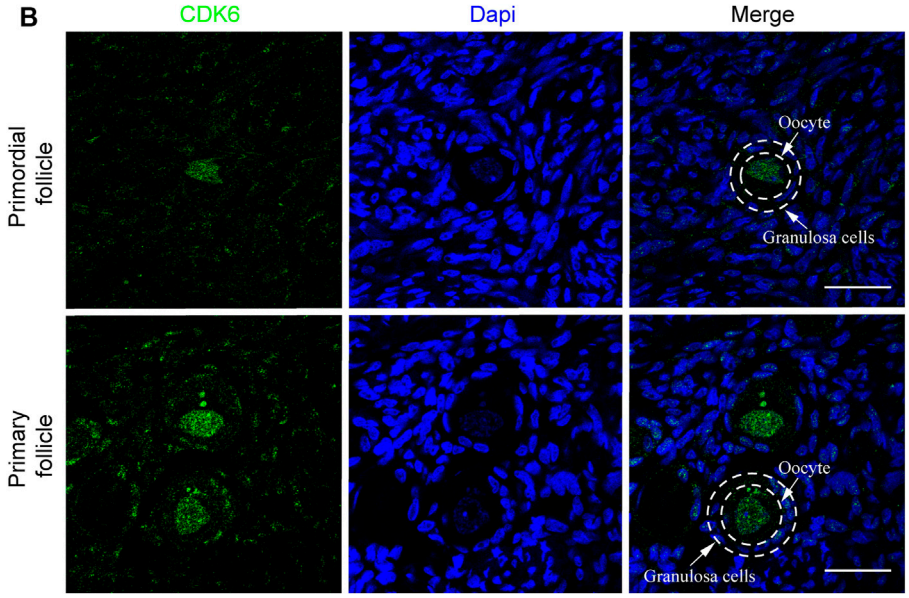

FIGURE 1
CDK6 mRNA and protein expression in human ovarian tissue. **(A)** Table with FPKM and *p* values of the gene expression of *CDK6* mRNA in human oocytes and granulosa cells of the primordial and primary follicle, and illustration of the morphology of the follicular stages. Significant *p*-values are noted in bold. **(B)** CDK6 protein expression in preantral follicle stages in human tissue. Negative controls are shown in Supplementary Figure S1. Scale bar: 20 μm.

CDK6 protein in both oocytes and granulosa cells of follicles at different developmental stages. Interestingly, a predominant nuclear distribution of CDK6 protein in the oocytes from both primordial and primary follicles was noted (Figure 1B). A no-primary-antibody negative control detected no specific staining (Supplementary Figure S1A).

BSJ reduced the level of CDK6 protein in mouse ovaries

To evaluate the role of CDK6 on primordial follicle activation, a pharmacological approach was applied using BSJ, as a selective CDK6 degrader. As a first indication of the significance of CDK6 in mouse ovarian tissue, immunofluorescence staining was performed on mouse *in vitro* cultured ovaries with or without BSJ at 1, 5, and 10 μM concentrations using an anti-CDK6 antibody. Likewise immunofluorescence staining in human ovarian tissue, CDK6 protein was distributed in both oocytes and granulosa cells, particularly in nuclei of oocytes, from follicles at primordial, primary and secondary follicle developmental stages (Figure 2A). Moreover, the expression of CDK6 protein in follicles at different developmental stages in cultured ovaries with BSJ was reduced compared to DMSO control group, as expected (Figure 2A). Immunofluorescence on mouse ovarian tissue without the primary antibody was shown (Supplementary Figure S1B) and detected no specific staining. Next, Western blotting analysis was applied to evaluate whether the expression of CDK6 protein was likewise decreased in mouse *in vitro* cultured ovaries with BSJ at 1, 5, and 10 μM concentrations compared to DMSO control groups. As expected, the results demonstrated that CDK6 protein level from *in vitro* cultured ovaries with BSJ were significantly lower than DMSO control group (Figure 2B; Supplementary Figure S4) (Table 1).

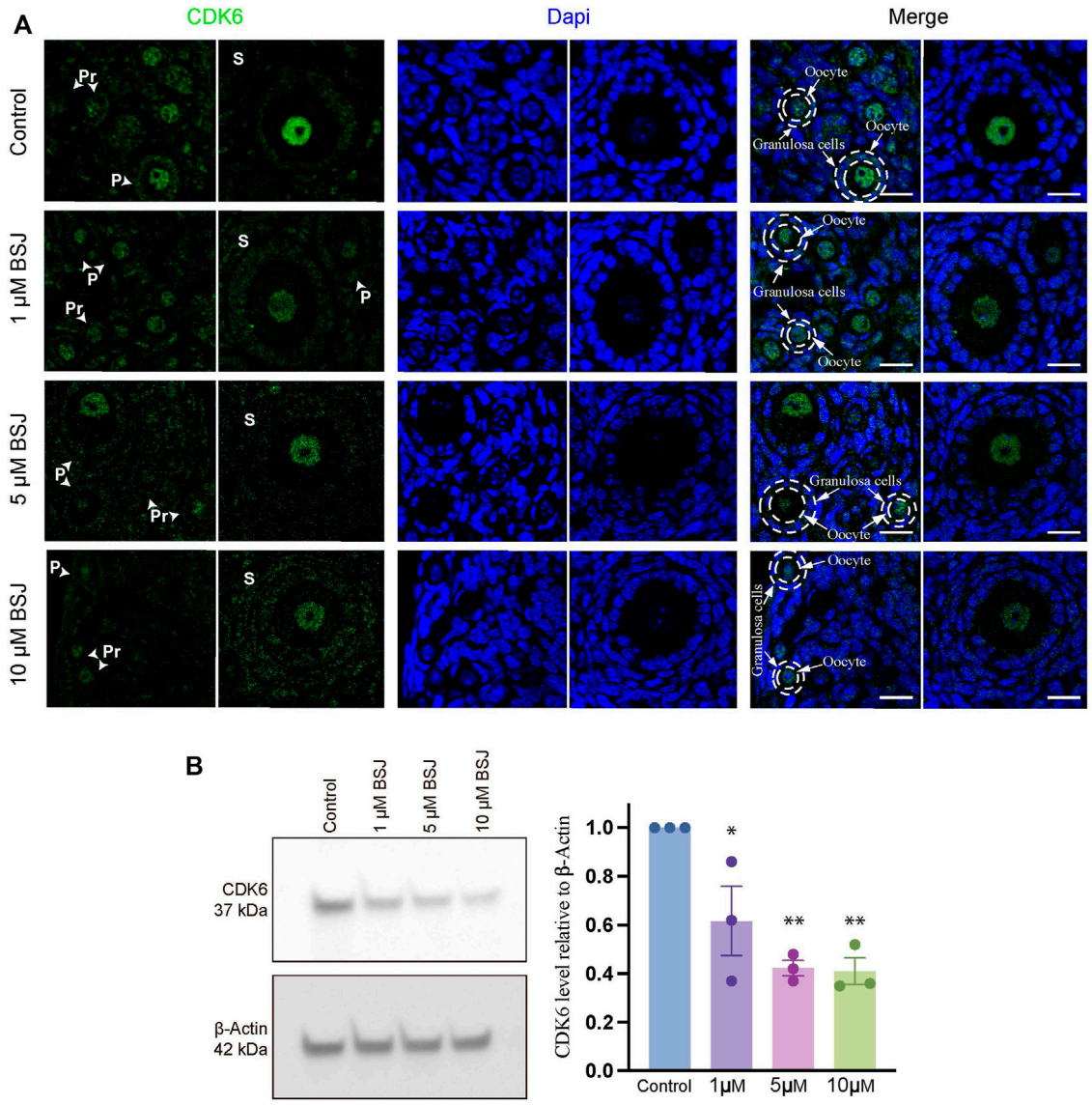

FIGURE 2
Nuclear expression of CDK6 in murine ovarian follicles after 5 days *in vitro* culture with 0–10 μM BSJ. **(A)** Immunofluorescent staining of CDK6 and counterstained with DAPI in primordial (Pr), primary (P) and secondary (S) Scalebar: 20 μm. Negative controls are represented in Supplementary Figure S1. **(B)** Representative western blotting with primary antibodies against CDK6 and beta-actin as loading control on ovaries *in vitro* cultured with different concentrations of BSJ (0–10 μM) for 5 days. Full-length membranes are represented in Supplementary Figure S4. Data reflects three independent biological replicates, $n = 12$, and are represented as mean ± SEM. Data are analyzed with one-way ANOVA followed by Bonferroni correction where the mean of each concentration is compared with the mean of the control. Statistically significant data are noted with asterisks, $*p < .05$, $**p < .01$.

TABLE 1 Western blotting data.

Bonferroni multiple comparison test	Adjusted *p*-value	Summary
Control vs. 1 μM	.0244	*
Control vs. 5 μM	.0023	**
Control vs. 10 μM	.0020	**

Statistically significant data are noted with asterisks, $*p < .05$, $**p < .01$, $***p < .001$, $****p < .0001$, ns, not significant.

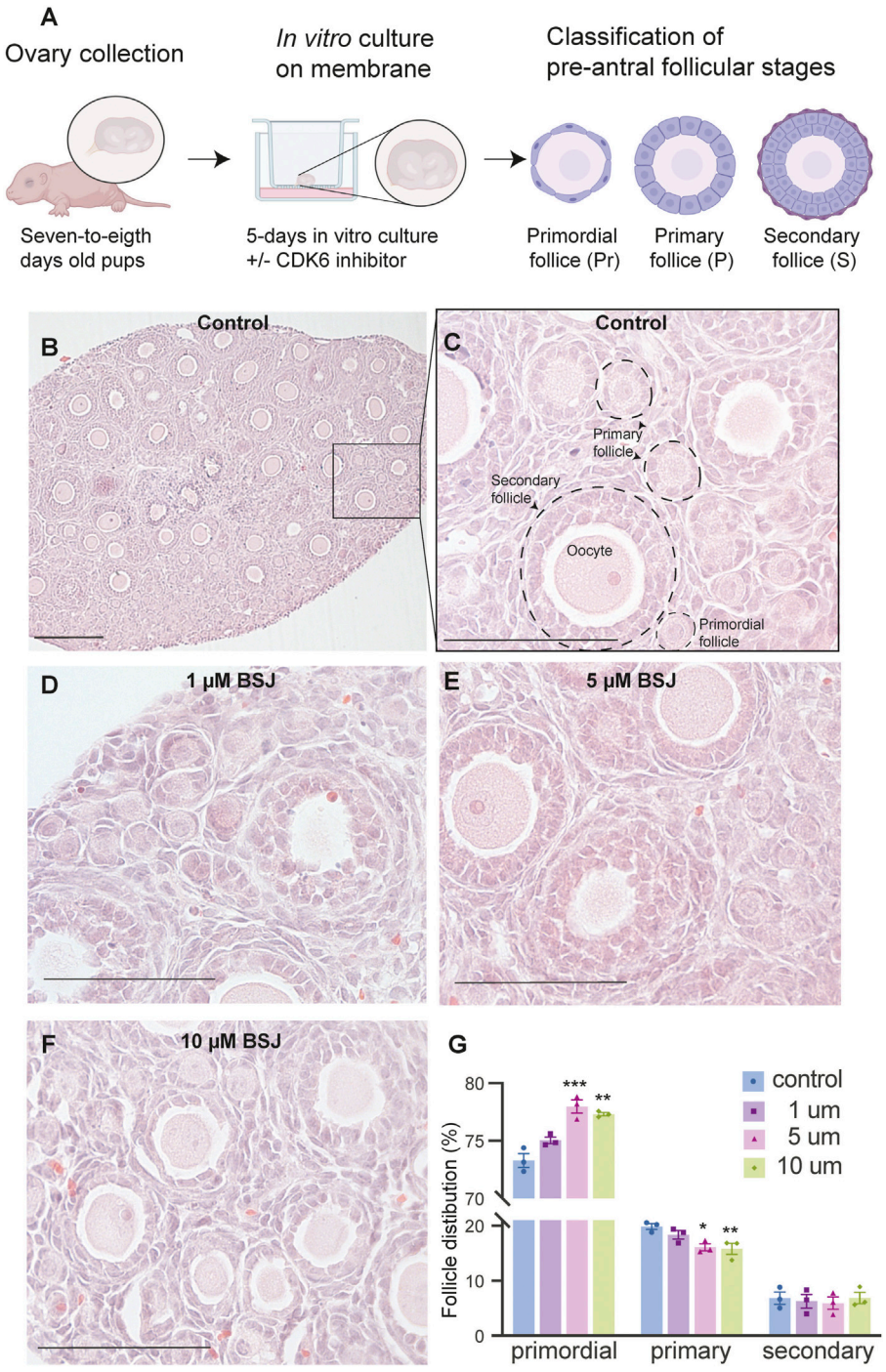

FIGURE 3
Inhibition of CDK6 suppress primordial follicle activation *in vitro*. **(A)** Schematic illustration of the experimental design and morphological characteristics of primordial (Pr), primary (P) and secondary (S) follicles. **(B)** Photomicrograph of whole ovary cultured 5 days *in vitro* in control medium. Scale bar: 100 μm. **(C)** The square defined in B is examined more closely to assess the follicular morphology, and follicular structures are defined. Scale bar: 50 μm. **(D–F)** Representative photomicrograph of H&E-stained ovary exposed to 1 μM BSJ **(D)**, 5 μM BSJ **(E),** and 10 μM BSJ **(F)**. Scalebar: 50 μm. Photomicrographs of whole ovaries and more representative sections of the ovary are shown in Supplementary Figure S2. **(G)** The distribution of primordial, primary and secondary follicles in ovaries exposed to 0–10 μM BSJ. Data are also represented in table 1 and includes 30–40 μM of BSJ, and photomicrographs of H&E-stained ovaries of these concentrations are shown in Supplementary Figure S2. For all concentration $n = 3$. The data is analyzed with one-way ANOVA followed by Bonferroni correction where the mean of each concentration is compared with the mean of the control. Statistically significant data are noted with asterisks, $*p < .05$, $**p < .01$, $***p < .001$, $****p < .0001$.

TABLE 2 Summary of follicle distribution in ovaries in vitro cultured with 0-40 μM BSJ. Data are represented as mean ± SEM. For all groups *n*=3. Data are analyzed with one-way ANOVA followed by Bonferroni correction. H&E images of whole and representative sections of the ovaries is shown in Supplementary Figures S2,S3.

	Control	1 μM	5 μM	10 μM	20 μM	30 μM	40 μM	*p*-value
Primordial follicle (%)	73.30 ± 0.59	75.04 ± 0.29	77.97 ± 0.58	77.30 ± .14	78.64 ± .17	79.91 ± 0.80	80.21±1.06	< .0001
Primary follicle (%)	19.87 ± 0.53	18.35 ± .078	16.10 ± 0.62	15.81 ± 1.01	13.57 ± .23	12.00 ± 0.50	11.89±1.04	< .0001
Secondary follicle (%)	6.84 ± 1.10	6.29 ± 1.25	5.94 ± 1.15	6.89 ±.03	7.90 ± .18	8.10 ± .30	7.90 ± .28	ns

TABLE 3 Summary of multiple comparison tests for primordial follicle.

Bonferroni multiple comparison test	Adjusted p-value	Summary
Control vs. 1 μM	.3712	ns
Control vs. 5 μM	.0005	***
Control vs. 10 μM	.0022	**
Control vs. 20 μM	.0002	***
Control vs. 30 μM	< .0001	****
Control vs. 40 μM	< .0001	****

Statistically significant data are noted with asterisks, *p < .05, **p < .01, ***p < .001, ****p < .0001, ns: not significant.

TABLE 4 Summary of multiple comparison tests for primary follicle.

Bonferroni multiple comparison test	Adjusted p-value	Summary
Control vs. 1 μM	.9588	ns
Control vs. 5 μM	.0148	*
Control vs. 10 μM	.0085	**
Control vs. 20 μM	.0002	***
Control vs. 30 μM	< .0001	****
Control vs. 40 μM	< .0001	****

Statistically significant data are noted with asterisks, *p < .05, **p < .01, ***p< .001, ****p < .0001, ns: not significant.

Reduction of CDK6 reduced primordial follicle activation in mouse ovaries

From above, it was evident that BSJ reduced levels of the CDK6 protein in ovaries. To functionally test of CDK6 inhibition would alternate follicle distribution *in vitro*, neonate ovaries were cultured on inserts for 5 days without or with BSJ at different concentrations (Figure 3A). Follicles were classified as primordial, primary and secondary follicles, as illustrated (Figure 3A). The morphology of granulosa cells and oocytes were persistent and follicles at different developmental stages were well organized in cultured ovaries at 1 and 5 μM concentrations of BSJ and DMSO control groups (Figures 3B–F). However, the shape of oocytes in growing follicles at 10–40 μM concentration of BSJ were irregular and less organized compared to the DMSO control group (Supplementary Figures S2, S3A). Interestingly, BSJ at 5, 10, 20, 30, and 40 μM concentrations significantly increased the percentage of primordial follicles (77.97 ± 0.58, 77.30 ± 0.14, 78.64 ± 0.17, 79.91 ± 0.80, and 80.21 ± 1.06) compared to DMSO control group (73.30 ± 0.59), while the percentage of primary follicles were significantly decreased in cultured ovaries with these concentrations (16.10 ± 0.62, 15.81 ± 1.01, 13.57 ± 0.23, 12.00 ± 0.50, and 11.89 ± 1.04) compared to DMSO control group (19.87 ± 0.53) (Figure 3G; Supplementary Figure S3B) (Table 2, Table 3, Table 4, and Table 5). The most significant effect of BSJ was observed at 5 μM (Figure 3G) However, the percentage of secondary follicles were not significantly changed

TABLE 5 Summary of multiple comparison tests for secondary follicle.

Bonferroni multiple comparison test	Adjusted p-value	Summary
Control vs. 1 μM	>.9999	ns
Control vs. 5 μM	>.9999	ns
Control vs. 10 μM	>.9999	ns
Control vs. 20 μM	>.9999	ns
Control vs. 30 μM	>.9999	ns
Control vs. 40 μM	>.9999	ns

Statistically significant data,*$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$, ns: not significant.

among groups (Figure 3G; Supplementary Figure S3B) (Table 2, Table 3, Table 4, and Table 5).

BSJ-induced CDK6 reduction enhanced apoptosis and ROS level

To evaluate the effect of CDK6 inhibition on apoptosis, TUNEL assay on cultured ovaries with and without BSJ at 1, 5, and 10 μM concentrations was performed. The results demonstrated that particularly, granulosa cells had undergone apoptosis compared to oocytes in all groups (Figure 4A). The proportion of apoptotic cells in cultured ovaries with BSJ at different concentrations increased in a dose dependent manner. Moreover, the proportion of apoptotic cells in cultured ovaries with BSJ at 5 and 10 μM concentrations (19.76 ± 2.35, 19.89 ± 2.98, respectively) was significantly higher than those of cultured in DMSO control group (7.16 ± 1.19) (Figure 4B) (Table 6, and Table 7). Negative control of TUNEL staining with no reacting enzyme on mouse ovarian tissue detected no apoptosis. (Supplementary Figure S1C).

In order to assess the effect of CDK6 inhibition on ROS level, as one of the important criteria to define the quality of oocytes and follicles, we investigated ROS levels in mouse *in vitro* cultured ovaries with or without BSJ at 1, 5, and 10 μM concentrations using the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescence assay. As expected, the ROS level in cultured ovaries with BSJ at different concentrations, was increased in a dose dependent manner (Figure 4C). However, the results showed no significant difference among ROS level of cultured ovaries with BSJ at 1 (1298 ± 125.6), 5 (1699 ± 248.5) and 10 (1734 ± 215.7) μM concentrations and DMSO control group (1046 ± 41.37) (Table 8 and Table 9).

As we observed that BSJ could reduce the activation of primordial follicles, and that cell quality appeared good in regards to apoptosis and ROS levels, a next step was to evaluate if there would be any differences in the classical P-AKT and AKT levels. We observed no differences in P-AKT or AKT in ovaries treated with BSJ compared to the DMSO control (Figures 4D–F), and therefore, it appears that BSJ-treatment does not involve the AKT pathway to detectable levels.

In vitro matured MII oocytes derived from exposed ovaries to BSJ resumed the first meiosis

One of the most important criteria to evaluate the quality of oocytes is to assess their meiotic competence. Therefore, we applied a two-step culture system for further analysis of oocyte quality in exposed ovaries to BSJ. First, the ovaries were cultured for 7 days in two groups as BSJ and DMSO control. Then the isolated secondary follicles were cultured in three-dimensional culture system using basic α-MEM (with no BSJ or DMSO) for 12 days, followed by ovulation induction and obtaining MII oocytes (Figure 5A). The morphology of follicles and which were isolated from cultured ovaries in BSJ were comparable to those of isolated from cultured ovaries in DMSO control groups (Figure 5B). Moreover, the secondary follicles in both groups had capability to grow up to antral follicle stage (Figure 5C) and there was no significant difference in the number of survived and degenerated follicles between two groups (Figure 5D, E) (Table 10). To examine and compare the development of follicles, we measured the diameter of follicles during 12 days culture period. The results showed that diameter of follicles in BSJ group at day 6 (135.3 ± 3.72) was significantly lower than that of control group (146.6 ± 2.97), which showed the effect of BSJ on size of follicles. However, the diameter of follicles at day 0 (72.49 ± 1.48) and 12 (274.5 ± 0.59) in BSJ group was not significantly different from those of control group at these days (71.00 ± 2.74 and 273.9 ± 4.24, respectively) (Figure 5F). After *in vitro* oocyte maturation, we showed that morphology of MII oocytes which were derived from cultured ovaries in BSJ were similar to those of derived from cultured ovaries in DMSO control groups, as MII oocytes had regular and round shape with normal polar body in both groups (Figure 5G). In order to

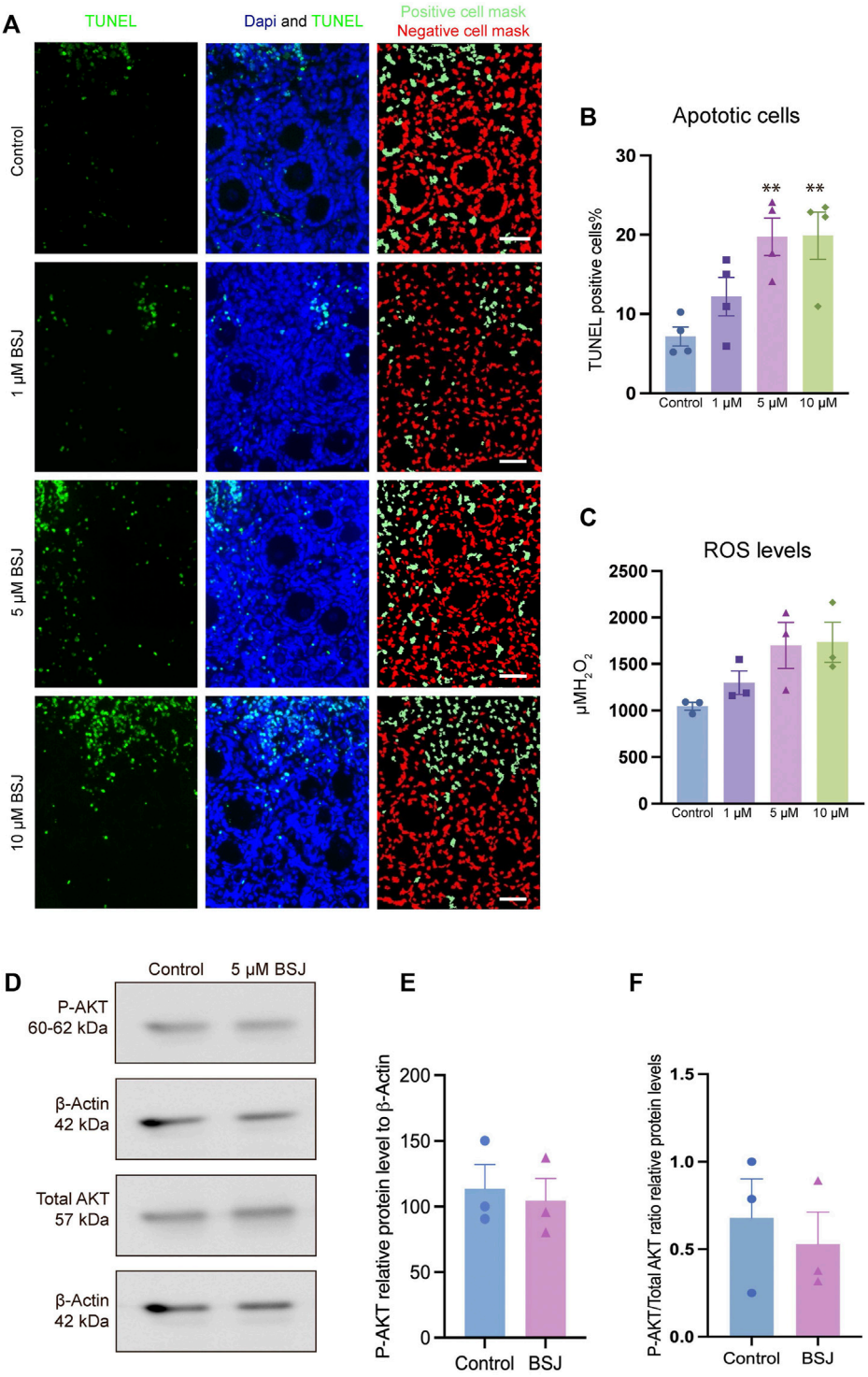

FIGURE 4
TUNEL and ROS quantification of ovaries cultured with different concentrations of BSJ (0–10 μM) *in vitro* for 5 days. **(A)** The number of apoptotic cells were detected with TUNEL staining. Row 1: TUNEL stain, Row 2: Merge of TUNEL and DAPI stain, row 3: Analysis of apoptosis quantification using an automated cell imaging system. Green cell mask represents cell positive for TUNEL stain, whereas red cell mask represents viable cell mask. Negative controls are represented in Supplementary Figure S1. **(B)** Quantification of percentage of cells positive for TUNEL stain. For all groups $n = 4$. **(C)** Quantification of ROS levels in ovaries *in vitro* cultured for 5 days with different concentration of (0–10 μM) BSJ. For all groups $n = 6$. **(B,C)** Data are analyzed with one-way ANOVA followed by Bonferroni correction where the mean of each concentration is compared with the mean of the control. Statistically significant data are noted with asterisks, $*p < .05$, $**p < 0.01$. **(D)** Representative Western blotting with primary antibodies against Phospho-AKT, AKT and beta-actin as loading control on ovaries *in vitro* cultured with 0 and 5 μM BSJ for 5 days. Full-length membranes are represented in Supplementary Figure S4. Data reflects three independent biological replicates, $n = 12$ in each group, and are represented as mean ± SEM. Data are analyzed with a two-tailed *t*-test.

TABLE 6 Number of apoptotic cells represented as mean ± SEM. *n*= 4.

	Control	1 μM	5 μM	10 μM
Apoptotic cells (%)	7.165 ± 1.196	12.19 ± 2.421	19.76 ± 2.356	19.89 ± 2.985

TABLE 7 TUNEL data.

Bonferroni multiple comparison test	Adjusted p-value	Summary
Control vs. 1 μM	.4604	ns
Control vs. 5 μM	.0073	**
Control vs. 10 μM	.0068	**

Statistically significant data are noted with asterisks, *p < .05, **p < .01, ***p < .001, ****p < .0001, ns, not significant.

TABLE 8 ROS data represented as mean ± SEM. *n*= 6.

	Control	1 μM	5 μM	10 μM
ROS levels (μM H2O2)	1046 ± 41.37	1298 ± 125.6	1699 ± 248.5	1734 ± 215.7

TABLE 9 ROS data.

Bonferroni multiple comparison test	Adjusted p-value	Summary
Control vs. 1 μM	> .9999	ns
Control vs. 5 μM	.0942	ns
Control vs. 10 μM	.0756	ns

analysis the maturation of oocytes, we measured the diameter of *in vitro*-matured MII oocytes and the results showed no significant difference between BSJ (48.40 ± 0.37) and control (48.61 ± 0.39) groups (Figure 5H). Moreover, MII oocyte maturation rate in BSJ group (53.94 ± 4.21) was not significantly different from that of control group (61.08 ± 2.02) (Figure 5I) (Table 10).

Discussion

In the present study, reduction of CDK6 using BSJ reduced primordial follicle activation, elucidating a role of CDK6 in transition from primordial to primary follicle stage. In our approach, we thought to accomplish a reduction of CDK6 activity, rather than a full inhibition, which was still very effective to reduce the activation of primordial follicles. It has been shown that reduction of CDK6 and cyclin D1 expression prevented proliferation of primordial germ cells in the porcine ovary due to inhibition of G1/S transition resulting in cell cycle arrest (Dai et al., 2021). Beside the role of CDK6 in cell cycle progression, CDK6 is also important in cell cycle independent functions (Malumbres et al., 2004; Hu et al., 2009; Hu et al., 2011; Laurenti et al., 2015; Scheicher et al., 2015). CDK6 can enhance vascular endothelial growth factor (VEGF) and angiogenesis (Kollmann et al., 2013). Recent studies showed that VEGF could activate primordial follicles through formation of blood vessels and induce angiogenesis (Nilsson et al., 2007; McFee et al., 2012; Komatsu and Masubuchi, 2020). Although the molecular mechanisms regulating primordial follicle activation are still being ambiguous, many studies have shown that PI3K/AKT signaling pathway can be the main signaling pathway to govern the primordial follicle activation (Li et al., 2021; Vo and Kawamura, 2021). Interestingly, it has been shown that overexpression of CDK6 increased the activation of PI3K/AKT signaling pathway in primary mouse hippocampal neurons, suggesting the potential link between CDK6 expression and PI3K/AKT signaling pathway (Zhang et al., 2021). In line with this, CDK6 inhibition reduced the activity of MAP-ERK and PI3K/AKT/mTOR signaling pathways in an acute myeloid leukemia cell line (Nakatani et al., 2021). IN summary, these studies support our

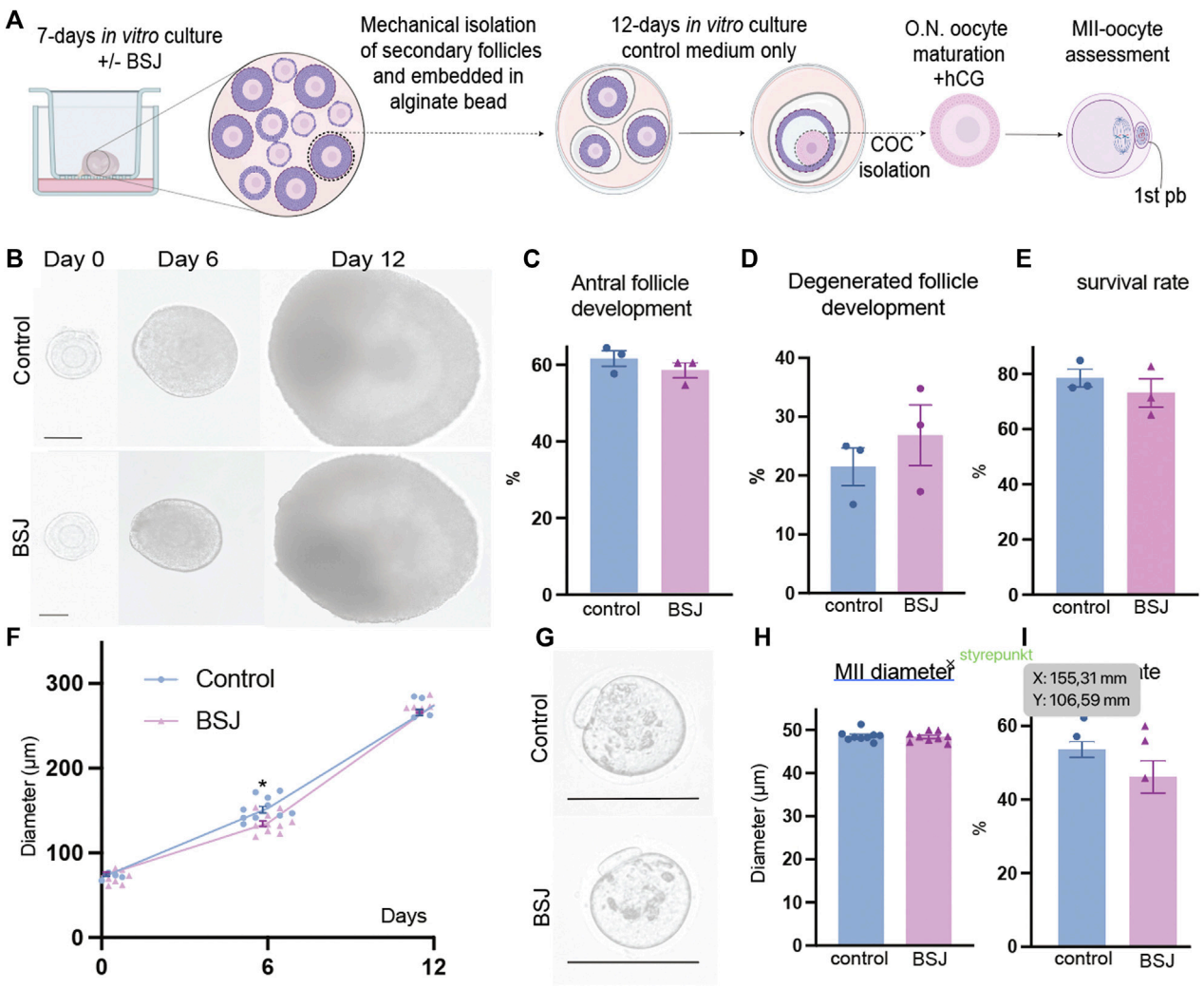

FIGURE 5
Oocytes exposed to BSJ during primordial follicle activation can still resume the first meiosis. **(A)** Schematic illustration of the experimental design of the *in vitro* strategy for developing MII oocytes from primordial follicles. **(B)** Photomicrographs of follicles during the 12-day three-dimensional follicle culture at day 0, 6, and 12. Scale bar: 50 µm and the respective diameters of the follicles at day 0, 6, and 12. For all groups $n = 10$. **(C)** Antral follicle development ($n = 22–29$). **(D)** Degenerated follicle development ($n = 12$). **(E)** Survival rate ($n = 36–47$). **(F)** Representative photomicrographs of MII oocytes. Scalebar: 50 µm. **(G)** MII diameter, $n = 9$. **(H)** MII rate $n = 21–29$. Data are represented as mean ± SEM and analyzed with a two-tailed *t*-test, $*p < .05$.

TABLE 10 Follicle and MII data.

	Control	5 µM	p-value
Antral follicle development	61.08 ± 2.029	58.06 ± 1.944	.3417
Degenerated follicle development	21.47 ± 3.195	26.87 ± 5.135	.4239
Survival rate	75.53 ±3.195	73.13 ± 5.135	.4230
MII diameter	48.61 ± 0.3985	48.40 ± 0.3796	.6984
MII rate	61.08 ± 2.029	53.94 ± 4.217	.2017

observations that inhibition of CDK6 could reduce the PI3K/AKT pathway, which would lead to reduce activation of primordial follicles. If note, and in contrast, it has been determined that CDK6 inhibition induced phosphorylation of AKT1S1 and increased the activation of mTORC1 signaling (Chang et al., 2022), but the details of how this is achieved is not known.

Our results showed that CDK6 inhibition increased apoptosis, particularly in granulosa cells, suggesting the possible effect of CDK6 inhibition on follicular or oocyte quality through induction of apoptosis. In line with this, it has been reported that immature *Cdk6*-deficient thymocytes show reducing in proliferation and induction of apoptosis (Hu et al., 2009). Moreover, the combination of CDK6 inhibition and a mitogen-activated protein kinase may result in cell cycle arrest and increasing in apoptosis a NRAS mutant melanoma mouse model (Kwong et al., 2012). Despite induction of apoptosis in this current study, CDK6 inhibition had no significant effect on ROS levels, which is known as one another marker for oocyte quality. In support of this, it was reported that CDK6 inhibition using Quercetin had no negative effect on viability of breast cancer and human adenocarcinoma cells, associating with induction of apoptosis (Yousuf et al., 2020). In contrast, it has been shown that CDK6 inhibition can impair antioxidants and significantly enhance ROS level (Chang et al., 2022). Moreover, it has been reported that CDK6 can regulate stabilization and activation of FOXM1, as a substrate of CDK4/6-Cyclin D complexes, leading to reduction of ROS production (Anders et al., 2011). IN summary, the effect of CDK6 inhibition might depend on the cell system used and also the specific functions of the cell population investigated.

These controversies guided this study to address the effect of CDK6 inhibition on oocyte quality, more extensively. Surprisingly, the *in vitro* cultured ovaries with CDK6 inhibitor had capability to produce competent *in vitro* matured MII oocytes, confirming no harmful effect of CDK6 inhibition on quality and meiotic maturation of oocytes. In consistent with our results, previous investigations on mice have determined that deletion of *Cdk6* could produce healthy animals with normal oocyte maturation, showing that *Cdk6* is not necessary for meiotic maturation of oocytes (Kohoutek et al., 2004; Adhikari et al., 2012; Risal et al., 2016). Moreover, Oqani et al. (2017) determined that inhibition of CDK6 by Palbociclib in porcine oocytes, did not affect cumulus expansion or formation of GVBD. Although, the *in vivo* functional role of CDKs during meiotic resumption of oocytes is still unravel, recent studies have shown that lacking of *Cdk6* in mice may be compensated by *Cdk1*, which can form active complexes with cyclins (Santamaria et al., 2007; Adhikari et al., 2012). In contrast, it has been reported that depletion of *Cdk6* in insects significantly reduces ploidy, arrested ovarian growth and oocyte maturation (Wu et al., 2018). Moreover, it has been shown that inhibition of CDK4/6 kinases in mice impaired spindle assembly, chromosome alignment and kinetochore-microtubule attachments following by increasing in progression of meiosis resulting in generation of aneuploid oocytes (Dong et al., 2021). However, there are many controversies between studies and the precise action of CDK6 during oocyte meiotic progression is still ambiguous, and awaits further investigations. In conclusion, the present study showed that CDK6 inhibition can regulate primordial follicle activation, with no effect on the quality and meiotic maturation of oocytes. However, the involved molecular mechanism of CDK6 inhibition in regulation of primordial to primary follicle transition is still unknown and requires further investigations.

Material and methods

Transcriptome data

The CDK6 gene expression data was extracted from two previous studies (Ernst et al., 2018, Ernst et al., 2017) using the large-scale data files http://users-birc.au.dk/biopv/published_data/ernst_2017 and http://users-birc.au.dk/biopv/published_data/ernst_et_al_GC_2017.

In these studies, the human ovarian cortical tissues were taken from three patients (26, 34 and 34 years old) undergoing oophorectomy before gonadotoxic treatment of a malignant disease. The patients normo-ovulatory, non-stimulated and with normal levels of reproductive hormones. In these global gene expression files FPKM values for all transcripts were calculated based on triplicate FPKM values using a one-sample *t*-test, based on a class comparison study of existing oocyte and granulosa cell transcriptomes from primordial (n = 539 follicles) and primary (n = 261) follicles collected from three patients. All transcripts were subsequently sorted according to their expression consistency across triplicates, as indicated by the one-sample *t*-test *p*-value (the cut-off value for inclusion in downstream analysis: $p < 0.2$ across triplicates). The CDK6 gene contribution in granulosa cells from primordial and primary follicles were collected and analysed using the global transcription lists (http://users-birc.au.dk/biopv/published_data/ernst_2017 and http://users-birc.au.dk/biopv/published_data/ernst_et_al_GC_2017) for 1) oocytes from primordial follicles 2) oocytes from primary follicles, 3) primordial follicles (oocytes with surrounding granulosa cells), and d) primary follicles (oocytes with surrounding flattened granulosa cells). Consistency in mean gene expression level (from the FPKM values) for all detected transcripts was quantified

by performing a *t*-test on patient triplicate samples from oocytes and granulosa cells from primordial and primary follicles, respectively. Consistency was ranked based on *p*-value, with a low *p*-value indicating a high degree of consistency in the mean FPKM across patient triplicates.

Animal

Female C57BL/6JRj mice and male CBA/JRj mice were housed under a 12-h light/dark cycle and breeding was performed to generate C57BL × CBA F1 mice in the biomedical animal facilities at Aarhus University. All procedures were approved by the Ethics Committee for the Use of Laboratory Animals at Aarhus University (permit number: 2020-15-0201-00757 to KLH).

In vitro ovarian culture

The ovaries were separated from 7 to 8 days old mice after cervical dislocation. The ovaries were then isolated from their surrounding tissues and washed with alpha-minimal essential medium (α-MEM, Gibco, Scotland, United Kingdom) supplemented with 10% fetal bovine serum (FBS, Gibco). The collected ovaries were transferred to inserts (pore size of 0.4 mm, 6.5 mm diameter, Corning, MA, United States) which were placed in 24-well plates at 37 C and in a humidified atmosphere of 5% CO2–95% air. The ovaries were cultured in α-MEM supplemented with 10% FBS; 5 mg/ml insulin, 5 mg/ml transferrin, and 5 ng/ml sodium selenite (1% ITS; Gibco Cat. No. 41400045); 1% penicillin–streptomycin solution (Thermo Fisher Cat. No. 15140122); and 100 mIU/ml rFSH or GONALf (Serono) for 5 or 7 days. In addition, BSJ-03-123 (TOCRIS, Batch No: 1A/242335) at 1, 5, 10, 20, 30, 40, and 50 μM concentrations (reconstituted in DMSO) or only DMSO (DMSO control group) were administered to the culture medium, which were replaced with fresh culture medium every other day during culture period. The cultured ovaries for 5 days were collected for histological assessment, TUNEL assay, immunofluorescent staining, ROS assay and western blotting. The cultured ovaries for 7 days were considered for follicle culture.

Histological assessment

In vitro cultured ovaries were fixed in paraformaldehyde 4% (PFA; Merck, 1.04005.1000) overnight at 4°C. Then, ovaries were dehydrated in a series of ethanol at room temperature (RT): 70%, 80%, 90%, 100% and the ovaries were cleared in xylene (VWR, 28.973.363) followed by infiltrating and embedding in paraffin at 60°C. The embedded tissues were then serially cut into 5 μM slices using Microtome (SLEE medical GmbH, cut6062) and stained by hematoxylin and eosin (H&E). For H&E staining, paraffin-embedded sections were deparaffinized at 60°C for 30 min followed by placing in xylene two times, 15 min in each. Next, the sections were rehydrated in a series of ethanol, placed in hematoxylin (Merck, 1.04302.01000) for 40 s, washed in water and transferred to eosin (Merck, 1.15935.0025) for 46 s, followed by dehydrating in a series of ethanol. The sections were then cleared in xylene and mounted using mounting medium (Sigma, 03,898). Follicle counting was conducted on every fifth section of entire ovaries. The follicles at different developmental stages were classified, previously. Briefly, the primordial follicles were defined as a single layer of flattened pre-granulosa cells surrounding an oocyte. The primary follicles were considered as a single layer of cuboidal granulosa cells surrounding an oocyte and the follicles contained two or more layers of granulosa cells were defined as secondary follicles. Only follicles containing a nucleus were counted to avoid recounting of the same follicle. An inverted microscope (Leica, BMI4000B) was used for assessment of the follicle stages.

TUNEL assay

A TUNEL (TdT-mediated dUTP-X nick end labeling) assay was performed for detection andquantification of apoptosis within ovaries affected by increasing concentration of BSJ using Cell Death Detection Kit (Sigma, 11684795910), according to the instruction of manufacture with few adjustments. Briefly, the cultured ovaries for 5 days, were fixed, dehydrated, embedded, and sectioned as previously described. Then, the slides were deparaffinized and rehydrated followed by permeabilizing using proteinase K. Next, the labelled sections with TdT were incubated for 2 h in a humidity chamber at 37°C, while the negative controls were omitted the TdT. Subsequently, the sections were counterstained with DAPI. The sections were analyzed immediately using fluorescent imaging (Molecular devices, PICO imageXpress) by calculating the number of apoptotic cells compared to total number of cells in sections of ovarian cortex.

Immunofluorescent staining

The immunofluorescence (IF) was performed for detecting intracellular proteins localization. The cultured ovaries were fixed, dehydrated, embedded and sectioned at 5 μm. The experiments were performed in three replicates and representative sections from the ovaries were included in each replicate. The slides with sections were deparaffinized, rehydrated and subjected to antigen retrieval with 0.01 M

sodium citrate buffer (pH 6.0) at high temperature (95–98°C) for 15 min. The slides were then rinsed thoroughly with PBS, permeabilized using 0.5% Triton 100X for 10 min and blocked with normal donkey serum in PBS (10%) for 30 min at room temperature. Next, the slides were incubated with CDK6 (1: 100, PA5-27978, Invitrogen) diluted in PBS containing 10% donkey serum, as primary antibody, overnight at 4°C. The next day, sections were washed in PBS followed by incubating with secondary antibody matching the serum block and primary antibody (Donkey Anti-Rabbit Alexa Flour 488 (1:300) for 1 h at RT. Then, the slides were washed in PBS, counterstained with DAPI for 3 min and mounted with fluorescence mounting medium (Dako, S3023). The sections were analyzed by an LSM510 laser-scanning confocal microscope using a ×63 C-Apochromat water immersion objective NA 1.2 (Carl Zeiss, Göttingen, Germany). The images were captured and assessed by Zen 2011 software (Carl Zeiss).

Western blotting analysis

In vitro cultured ovaries were rinsed in PBS, pooled and lysed in RIPA buffer contained protease and phosphatase inhibitors. Concentration of protein was detected by the Lowry method with BSA quantification as a standard, and 20 µg was used per lane. Samples were then heated for 10 min at 70°C, before electrophoresis. Western blotting was performed using electrophoresis on SDS-PAGE gels and transferring onto PVDF membranes was performed for 1.5 h at 70 V. Membrane blocking was applied using Tris-buffered saline (pH = 7.6) supplemented with 0.1% Tween-20 and 5% non-fat dry milk for 1 h at room temperature. Next, the membranes were incubated overnight with CDK6 (1:500, PA5-27978, Invitrogen), Phospho-AKT (Ser473) (1:2000, Cell signaling technologies, 4060) and AKT (1:1000, Cell Signaling Technology, 9272) as primary antibody and beta-actin (1: 3000, Sigma, A5441), as the loading control. The membranes were then washed with TBST (0.1% tween 20) followed by incubating with matching secondary antibody (1:3000, ThermoFisher Scientific, 65-6120 and 61-6520) for 1 h at room temperature. Visualization was performed using a chemiluminescence-based substrate and ImageJ was applied for quantification of the bands.

ROS assay

The ovaries were washed in PBS and incubated with 40 mmol/L Tris–HCl buffer (pH = 7.0) containing 5 mmol/L DCFH-DA (Sigma) at 37°C for 30 min. The sample were rinsed again with PBS and lysed in 10 mM Tris-HCl containing 20 mM EDTA and 0.25% Triton 100X, followed by centrifuging at 4°C and 10,000 × *g* for 20 min. Finally, the supernatants were loaded into a black 96-well plate and assessed using a spectrofluorometer at 488 nm excitation and at 525 nm emissions.

Encapsulation and three-dimensional *in vitro* culture of isolated secondary follicles

The ovaries were divided in two groups and cultured with BSJ at 5 µM concentration or DMSO for 7 days followed by isolation of secondary follicles (diameter between 60–82 µm) using insulin-gauge needles under a stereomicroscope (Leica, MZ75). The isolated follicles were then encapsulated using sodium alginate as previously described. Briefly, sodium alginate solution was prepared at a concentration of 0.5% (w/v) followed by filtering the solution. Next, each follicle was placed in a droplet of sodium alginate (7 µl), followed by transferring the droplets into a cross-linking solution (50 mM CaCl2 and 140 mM NaCl). The alginate beads were then washed using α-MEM medium. Finally, encapsulated follicles were cultured in basic α-MEM (with no BSJ or DMSO) supplemented with 10% FBS; 5 mg/ml insulin, 5 mg/ml transferrin, and 5 ng/ml sodium selenite (1% ITS; Gibco Cat. No. 41400045); 1% penicillin–streptomycin solution (Thermo Fisher Cat. No. 15140122); and 100 mIU/ml rFSH or GONALf (Serono) under mineral oil at 37 C with 5% CO2 for 12 days. Half of the culture media was changed with fresh media every other day, during culture period. Photomicrographs of the follicles were conducted using an inverted microscope (Leica, BMI4000B) at days 0, 6, and 12.

In vitro ovulation induction

At the end of individual follicle culture period, antral follicles were mechanically released from sodium alginate beads using gauge 25 needles. Next, cumulus-enclosed oocytes (CEOs) were isolated from antral follicles released from sodium alginate beads, carefully, with no damage to the oocytes. Ovulation was then induced by transferring the CEOs to micro drops of α-MEM supplemented with 10% FBS; 5 mg/ml insulin, 5 mg/ml transferrin, and 5 ng/ml sodium selenite (1% ITS; Gibco Cat. No. 41400045); 1% penicillin–streptomycin solution (Thermo Fisher Cat. No. 15140122); 100 mIU/ml rFSH or GONALf (Serono) and 10 IU/ml hCG (Serono) under mineral oil at 37 C with 5% CO2 for 14 h.

Statistical analysis

Data analysis was performed using GraphPad Prism software version 8 (GraphPad Software, San Diego, CA, United States).

Data are shown as means ± standard error of the mean (SEM). Multiple group comparisons were performed using one way ANOVA followed by *post hoc* Tukey's test. For all analyses, *p* values below .05 were considered to indicate statistically significant differences.

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.

Ethics statement

The animal study was reviewed and approved by All procedures were approved by the Ethics Committee for the Use of Laboratory Animals at Aarhus University (permit number: 2020-15-0201-00757 to KL-H).

Author contributions

SA-N, AM-S, MA, and KL-H conceived the study. SA-N, JJ, AH, and MA performed experiments and analysed data. JM drafted all figures and did statistic analysis. MA, SA-N, and KL-H wrote the manuscript. All authors approved the final manuscript.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.1036917/full#supplementary-material

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