THE EXPANDING NETWORK OF P53 SIGNALING: REACHING TO THE UNKNOWN OF CANCER

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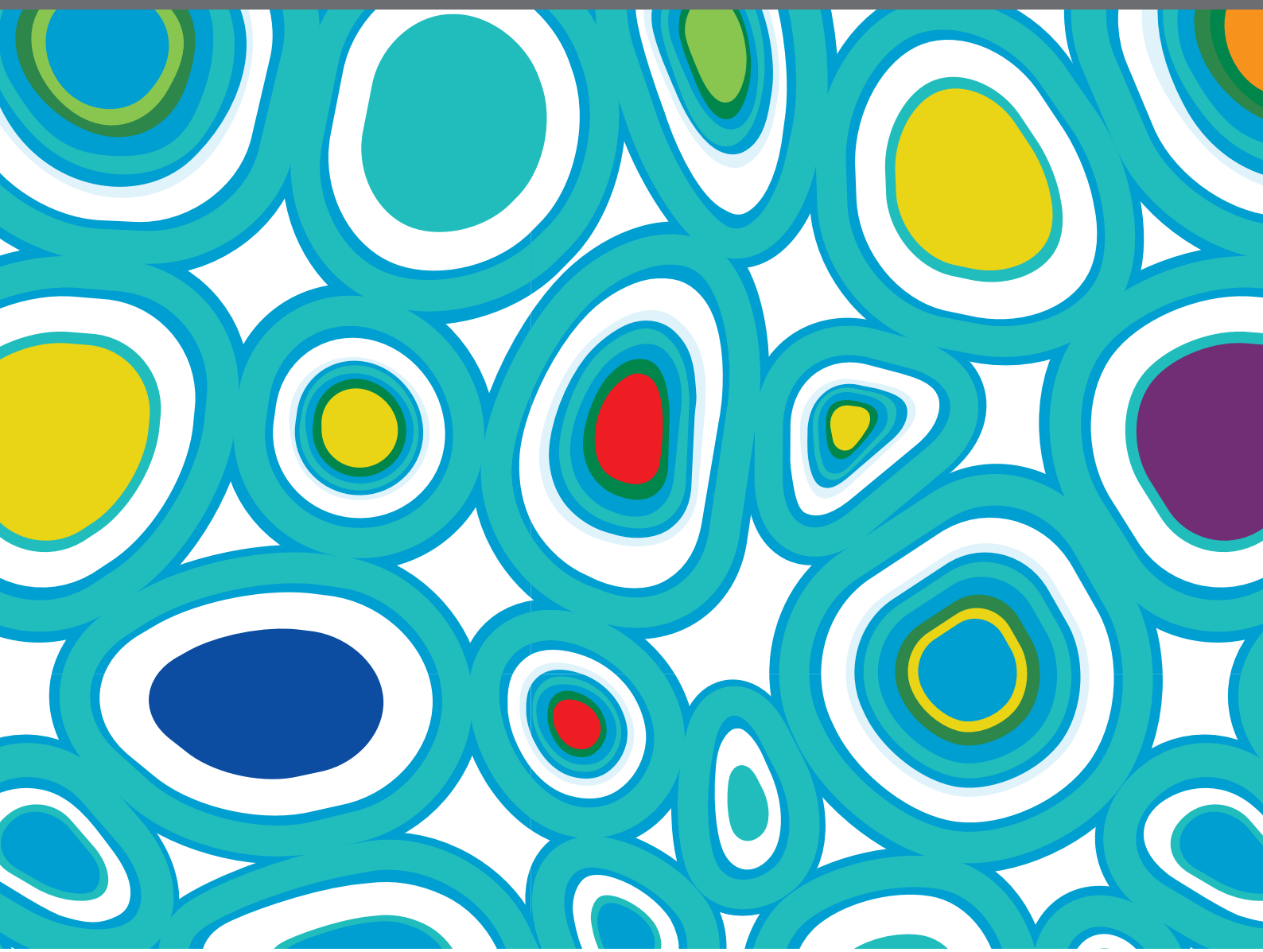

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THE EXPANDING NETWORK OF P53 SIGNALING: REACHING TO THE UNKNOWN OF CANCER

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Editorial: The expanding network of p53 signaling: Reaching to the unknown of cancer

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Editorial on the Research Topic
The expanding network of p53 signaling: Reaching to the unknown of cancer

Four decades of p53 research since the discovery of this tumor suppressor have demonstrated that p53 prevents tumorigenesis by maintaining genomic stability and eliminates cancer cells by inducing cell growth arrest, necrosis, apoptosis, and ferroptosis (Levine and Oren, 2009; Levine, 2020). p53 activity is tightly controlled by various mechanisms. One master controller is the E3 ubiquitin ligase MDM2 that is encoded by a p53 target gene and inhibits the anti-tumor functions of p53 by alleviating its protein translation, blocking its transcriptional activity, and promoting its ubiquitination and proteolytic degradation (Lu, 2017; Zhou et al., 2017). Overcoming this negative feedback inhibition is crucial for p53 activation in response to different stress signals, including oncogenic stress, DNA damage stress, nucleolar stress, and nutrient restrictions (Zhou et al., 2015; Hafner et al., 2019). Also, p53 can be inhibited by other negative modulators, and thus, inactivating these modulators can lead to p53 activation as well (Zhou et al., 2017; Hafner et al., 2019). However, there are still numerous remaining questions in the p53 field. Is the canonical MDM2-p53 feedback circuit a really druggable target for the development of new anti-cancer therapies? What more could we learn about "gain of functions" (GOFs) of p53 missense mutants? Are there more molecular insights into the role of p53 in the maintenance of metabolic homeostasis and the prevention of cancer-induced metabolic remodeling? How do wild-type (wt) and mutant (mt) p53s regulate the immune response, and would generating mt p53 vaccine an effective and feasible approach for developing anti-cancer therapy? These outstanding questions are partly addressed in several nicely written review articles and novel research studies that are collected in this specific issue as briefly introduced below.

Blocking the MDM2-p53 feedback loop has been considered as a promising strategy to treat cancers harboring wt p53 for decades, although it has been quite challenging as

there is not an applicable drug targeting this loop as an anti-cancer therapy in clinic use. In the issue, a review article by Kung et al. gracefully described a canonical mechanism by which oncogenic stress induces p53 activation with some new information and thoughts. Specifically, the oncogenic c-MYC or RAS signaling induces the expression of ARF, an alternate open reading frame encoded by CDKN2A, which in turn activates p53 by interacting with and inhibiting MDM2 activity. Importantly, they also summarized several potential therapeutic strategies targeting the ARF-MDM2-p53 cascade, including small molecules, peptides, and the proteolysis targeting chimera (PROTAC) tactic. In a research study, Han et al. reported that the PARP inhibitor olaparib, a targeted therapy for cancers with BRCA1/2 mutations or homologous recombination deficiencies, can induce p53 activation *via* RPL5/RPL11-mediated inhibition of MDM2 by triggering nucleolar stress, demonstrating an additional action mode of PARP inhibitors by targeting the nucleoli and activating the p53 pathway.

The p53-encoding gene, *TP53*, is the most frequently mutated gene in human cancers. The cancer-derived mutations of p53 include missense, frameshift, truncation, and deletion. Most of the p53 mutants are missense mutations that often occur in the DNA-binding domain of the p53 protein. These mutants not only lose their tumor inhibitory activity, but also exert a "dominant-negative" effect on the functions of wt p53. Remarkably, several hotspot mutants, such as mt p53-R175H, G245S, R248W/Q, R249S, R273H/C, and R282W, acquire GOFs to further promote tumor growth *via* diverse mechanisms (Freed-Pastor and Prives, 2012). Although these mutants usually lack the DNA-binding ability, they can indirectly regulate gene transcription by either binding to other transcription factors or modulating epigenetic modifications. Also, they can regulate other cellular processes through protein-protein interactions (Sabapathy and Lane, 2018; Zhou et al., 2019). As described in a review by Madrigal et al. in this issue, mt p53 regulation of microRNA expression involves both transcription-dependent and -independent mechanisms. Mt p53 was recently found to associate with replicating chromatin and PARP1 to facilitate aberrant DNA repair (Xiao et al., 2020). An interesting study by Annor et al. in this issue demonstrated that oligomerization of mt p53-R273H is not required for its chromatin association, though oligomerization of wt p53 is indispensable for its tumor suppressive activity. In a prospective essay by von Grabowiecki et al., the authors proposed a provocative idea that mt p53 might promote endosomal trafficking of a plethora of proteins involved in tumorigenesis and cancer progression by regulating Rab11-FIP1, which is supported by some recent studies as cited in this article and will await further validation.

Over the past years, growing evidence has revealed the crucial role of p53 in the maintenance of metabolic homeostasis and the prevention of cancer-associated metabolic remodeling (Labuschagne et al., 2018; Liu et al., 2019). Mammalian target of rapamycin (mTOR), an evolutionarily conserved serine/threonine protein kinase, serves as a central regulator by linking cellular nutrient status to cell growth. In this issue, Cui et al. offered a comprehensive review on the progresses of recent studies on the coordinated regulation of p53 and mTOR pathways in response to the physiological and genotoxic conditions. This is further consolidated by another review by Nagpal and Yuan, who elegantly collected numerous previous and new findings on the role of basally expressed p53 in restraining anabolic metabolism to prevent fast cell proliferation under non-stress conditions. In accordance, the tumor suppressive function of p53 has been also attributed to its activity to regulate glucose metabolism, lipid metabolism, amino acid metabolism, and iron metabolism in cancer cells, which are nicely illustrated in a review by Yu et al. Moreover, p53 has been shown to be involved in the regulation of recycling and clearance of metabolites, nutrients, and cellular debris. This line of information on wt and mt p53's new functions is systematically reviewed by Rahman et al. They offered another comprehensive and updated view on the roles of wt and mtp53s in the regulation of autophagy signaling and provided new insights into the therapeutic potential by modulating p53-mediated autophagy.

The roles of wt and mt p53s in the regulation of inflammatory and immune responses have been a hot topic recently. To update this area of research, this issue has also collected several review and research articles. For instance, the review article by Nagpal and Yuan described that basally expressed p53 is required for the maintenance of immune homeostasis. In addition, Shi and Jiang offered a detailed review on various mechanisms underlying wt and mt p53 regulation of inflammation and immunity. For example, p53 prevents inflammation-associated cancer development by suppressing NF-κB and STAT3 signaling pathways (Gudkov and Komarova, 2016; Wormann et al., 2016), while mtp53 antagonizes the STING/TBK1/IRF3 pathway, resulting in tumor evasion of immune surveillance (Ghosh et al., 2021). Moreover, a research article by Zhang et al. showed that p53 mutation is associated the increased production of chemokines, leading to infiltration of different immunocytes in breast cancer. This suggests a complex tumor microenvironment in mt p53-harboring cancers. The development of vaccines targeting p53 has been an old, yet unsolved, topic, as both wt and mtp53 epitopes can be presented on the cell surface for T cell recognition (Houbiers et al., 1993). To update this interesting research area, Zhou et al. offered a thoughtful review on the recent progresses of vaccination of p53 or its peptides and discussed the possibility and application of p53-targeting vaccines to cancer treatment. Collectively, these review and research articles as published in this issue not only show recent progresses in various regulations and roles of wt and mt p53 in cancer development, progression,

and immunology, but also provide more new insights into the p53 anti-cancer functions and mt p53's oncogenic activities. Importantly, these articles also offer new thoughts and suggestions for targeting mt p53 as anti-cancer therapies, such as new potential approaches for developing p53 vaccines.

Although the canonical functions of p53 as a key regulator of cell cycle, DNA repair, and apoptosis have been well documented, increasing studies have been continuingly unveiling novel roles of p53 in metabolic remodeling, immune surveillance, and cancer therapy, making this magic molecule as the most attractive research target as well as a promising therapeutic target for developing anti-cancer therapies in the future. We are deeply grateful to these authors who have made great efforts to our better understanding of new functions of p53 and new insights into this still mysterious molecule by contributing their comprehensive review and elegantly-designed research articles to this special issue.

Author contributions

XZ drafted the manuscript. XZ, JHJ and HL revised the manuscript. All authors contributed to the article and approved the submitted version.

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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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MINI REVIEW
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A Different Facet of p53 Function: Regulation of Immunity and Inflammation During Tumor Development

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As a key transcription factor, the evolutionarily conserved tumor suppressor p53 (encoded by *TP*53) plays a central role in response to various cellular stresses. A variety of biological processes are regulated by p53 such as cell cycle arrest, apoptosis, senescence and metabolism. Besides these well-known roles of p53, accumulating evidence show that p53 also regulates innate immune and adaptive immune responses. p53 influences the innate immune system by secreted factors that modulate macrophage function to suppress tumourigenesis. Dysfunction of p53 in cancer affects the activity and recruitment of T and myeloid cells, resulting in immune evasion. p53 can also activate key regulators in immune signaling pathways which support or impede tumor development. Hence, it seems that the tumor suppressor p53 exerts its tumor suppressive effect to a considerable extent by modulating the immune response. In this review, we concisely discuss the emerging connections between p53 and immune responses, and their impact on tumor progression. Understanding the role of p53 in regulation of immunity will help to developing more effective anti-tumor immunotherapies for patients with *TP53* mutation or depletion.

Keywords: p53, immune, inflammation, tumor microenvironment (TME), innate and adaptive immune response

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INTRODUCTION

As an intensively studied protein, the fame of p53 mainly stemming from its role as a tumor suppressor which is activated when responding to stress signals such as genotoxic damage, or nutrient deprivation (Lowe et al., 2004; Vousden and Lane, 2007; Levine and Oren, 2009). Mutations of p53 always accompanied dysregulation of metabolism, migration, and invasion, all of which ultimately result in the development of clinical tumors and an ever more aggressive malignancy (Hanahan and Weinberg, 2011; Jiang et al., 2013; Schwitalla et al., 2013; Labuschagne et al., 2018). Cancer cells can be recognized and destructed by innate and adaptive immune effector cells, a process that is known as cancer immunosurveillance (Zitvogel et al., 2006). In recent years, various studies have indicated that p53 can also control tumor-immune system crosstalk

(Watanabe et al., 2014; Guo and Cui, 2015; Blagih et al., 2020b). p53 loss in tumors provokes an altered myeloid and T cell responses. Specifically, p53 loss increases myeloid infiltration through enhanced secretion of cytokines (Blagih et al., 2020b). Morever, dysfunction of p53 under certain circumstance reprograms the components of tumor microenvironment (TME), leading to an altered immunologic milieu which exacerbates tumor progression. Here, we review the latest understanding of p53 in regulating the immune response during tumor development.

p53 REGULATION OF INFLAMMATION

Besides the capability of governing cellular homeostasis to curb tumourigenesis, accumulating observations suggest that p53 also plays the role in inflammatory reactions (Gudkov et al., 2011; Cooks et al., 2014). Chronic inflammation creates a potential cancer-promoting condition (Karin, 2006; Mantovani et al., 2008). In inflamed tissues, cytokines or inflammatory mediators can activate several transcription factors such as NF-κB and Signal Transducer and Activators of Transcription 3 (STAT3) which are critical in promoting cancer initiation. The activation of NF-κB and STAT pathways results in the enrichment of ROS in TME which ultimately prompts chronic inflammation (Trinchieri, 2012). Accumulating evidence strongly indicate that p53 dysfunction in tumors can enhance chronic inflammation and then promote tumor progression. Below, we discuss the role of p53 in inflammation.

NF-κB and p53

Chronic inflammation enhances the risk of cancer. As the crucial transcription factor, NF-κB is constitutively activated in most tumors. p53 and NF-κB pathways play crucial roles in response to various stresses and the NF-κB activity usually shows an antagonistic relationship with that of p53 (Kawauchi et al., 2008a; Ak and Levine, 2010; Gudkov et al., 2011). In contrast to p53 whose canonical role is growingly restrictive, NF-κB vastly promotes cell survival and inflammation. NF-κB and p53 have an extensive crosstalk in numerous cancers. Specifically, chronically inflamed and malignant tissues are always accompanied by constitutive activation of NF-κB where the p53 function is repressed by persistent infections or tissue irritating factors (Webster and Perkins, 1999; Schneider et al., 2010; Son et al., 2012; Natarajan et al., 2014). Mice with intestinal epithelial cell (IEC)-specific p53 deficiency do not initiate intestinal tumorigenesis, but significantly enhance carcinogen-induced tumourigenesis by promoting the establishment of an NF-κB-dependent inflammatory microenvironment that increases intestinal permeability and further invasion and metastasis (Schwitalla et al., 2013). Moreover, activated p53 acts as a suppressor directly suppressing the transcriptional activity of NF-κB, and aberrant inflammation can enhance tumor development when p53 is lost (Kawauchi et al., 2008a,b; Son et al., 2012; Gudkov and Komarova, 2016; Uehara and Tanaka, 2018).

Intriguingly, the reciprocal activation of p53 and NF-κB has been also found in certain cases (Lowe et al., 2014). It has been reported that p53 and NF-κB co-regulate the induction of pro-inflammatory genes, such as IL-6 and CXCL1, in human macrophages to drive the induction of pro-inflammatory cytokines (Lowe et al., 2014). Moreover, the activation of NF-κB promotes the secretion of numerous inflammatory cytokines and chemokines in senescent cells with highly activated p53 (Rodier and Campisi, 2011; Davalos et al., 2013).

As the most frequently genetic alterations in cancer, p53 mutations exist in over half of human cancers. However, many p53 mutants (mutp53) gain new activities to augment pro-inflammatory and survival properties, termed gain-of-function (GOF). Several studies have shown that GOF mutp53 can activate some of the NF-κB target genes (Cooks et al., 2013; Di Minin et al., 2014; Rahnamoun et al., 2017). For example, Cooks et al. (2013) demonstrated that mutp53 prolong NF-κB activation, leading to a significant proinflammatory activity and promoting colitis-associated colorectal cancer in mouse model. Di Minin et al. (2014) reported that mutp53 in cancer cells reprogram NF-κB and JNK activation in response to TNFα through the binding and interfering the tumor suppressor RasGAP Disabled 2 Interacting Protein (DAB2IP) in the cytoplasm. Mutp53 can also interact with NF-κB directly, enhancing RNA polymerase II recruitment in response to chronic TNF signaling which shapes the enhancer landscape and oncogenic gene expression (Rahnamoun et al., 2017). Therefore, inhibition of NF-κB to restore wild-type (WT) p53 function or reactivation of WT p53 in the context of mutp53 would be a very attractive target for cancer therapy.

Small Molecule Modulators Simultaneously Activate p53 and Inhibit NF-κB

As mentioned above, killing strategies that directly target the p53 and NF-κB pathways can be utilized to improve cancer therapy (Cheok et al., 2011; Khoo et al., 2014; Muller and Vousden, 2014). Several moleculers targeting both pathways have been indentified and some of which are already in clinical trials. For example, anti-malaria drug quinacrine was identified to have the ability to kill cancer cells by simultaneously inhibiting NF-κB and activating p53 (Gurova et al., 2005). Quinine and other aminoacridine derivatives mimic DNA damage, are non-genotoxic, and have good therapeutic potential for cancer in mouse xenograft models. This is noteworthy because anticancer drugs such as cisplatin induce p53 by forming covalent DNA adducts. r-Roscovitine, another small molecule, targets multiple signaling pathways simultaneously and prevents tumor growth. It activates p53 while blocking NF-κB activity and has shown its anticancer properties in phase II clinical trials (Lu et al., 2001; Dey et al., 2008). Interestingly, r-Roscovitine was originally developed as a cell cyclin-dependent kinase (CDK) inhibitor, which was shown to inhibit MDM2 expression and stabilize p53 (Lu et al., 2001). r-Roscovitine downregulates NF-κB activation in response to TNF-α and IL-1 by inhibiting IκB kinase (IKK) activity. It also inhibits the phosphorylation of p65 at Ser536 *via* IKK, which is required for nuclear localization. At the transcriptional level, r-Roscovitine inhibits the transcription of NF-κB-regulated genes

such as MCP-1, ICAM-1, COX2, FLIP, and IL-8 (Dey et al., 2008). Nutlin is the first Mdm2 antagonist reported to inhibit the p53-Mdm2 interaction and was shown to inhibit tumor growth in mouse models (Vassilev et al., 2004; Tovar et al., 2006). It was shown that Nutlin also strongly inhibits the protein expression of NF-κB target genes ICAM-1 and MCP-1, depending on p53 status (Dey et al., 2007). Clearly, more research is needed to better understand the mechanisms behind these drugs and to find more small molecules with higher specificity to activate p53 and inhibit NF-κB.

p53 and Signal Transducer and Activators of Transcription Pathways

Signal transducer and activators of transcription family is a group of transcription factors that regulate cytokine-dependent inflammation and immunity (Grivennikov et al., 2010). Constitutively activated STATs, especially STAT3, induce and maintain a protumourigenic inflammatory microenvironment to stimulate the initiate and survival of malignant cells (Catlett-Falcone et al., 1999; Mantovani et al., 2008; Grivennikov et al., 2009). p53 regulates inflammation response through STAT3 that is activated by inflammatory cytokine IL-6. And, p53 loss in pancreatic cancer results in activated STAT3 phosphorylation, which is initiated by IL-6 (Wormann et al., 2016). Like NF-κB, STAT3 binds to the p53 promoter directly to inhibit p53 transcription, limiting its canonical tumor suppressor function. Blocking STAT3 activates expression of p53, leading to p53-dependent tumor cell apoptosis (Niu et al., 2005). It has been shown that tumor cells dependent on long-term STAT3 signaling are more sensitive to STAT3 inhibitors than normal cells (Yu and Jove, 2004). Thus, STAT3 proteins can be targeted as novel cancer therapeutics, and more effective and selective STAT inhibitors can be expected to be developed in the future.

Besides the reciprocal relationship of STAT3 and p53, it has also been reported that inactivation of p53 in macrophages results in elevated levels of total and phosphorylated STAT1, thereby increases the production of proinflammatory cytokines (Zheng et al., 2005). Furthermore, p53 stimulates Treg cell differentiation *via* direct interaction with STAT5 (Park et al., 2013). Therefore, it is likely that p53 can balance the activity of various STAT pathways to impact host immune response.

CELLULAR CONSTITUENTS OF THE TUMOR MICROENVIRONMENT

Emerging studies suggest that tumor cell growth and invasion are markedly affected by tumor microenvironment (TME) (Kerkar and Restifo, 2012; Swartz et al., 2012). The TME contains not only cells but also signaling molecules, extracellular matrix, and mechanical cues. The immunological landscape of TME is shaped by all these cellular and molecular components that support neoplastic transformation, protects the cancer cells from host immunity, and provides niches for metastasis. Besides the cell-autonomous effects of p53, emerging evidence show that p53 can also have effects on neighboring cells, i.e., non-cell-autonomous activities of p53 (Bar et al., 2010; Lujambio et al., 2013). Thus, better understanding the function of p53 in TME may be potentially used to tailor personalized therapies for patients with tumors bearing p53 mutations.

Cancer-Associated Fibroblasts

In the TME, cancer-associated fibroblasts (CAFs) play an important role in modulating tumor progression and metastasis (Ohlund et al., 2014; Kalluri, 2016). In CAFs of highly inflamed cancers, p53 mutations are frequently detected (Patocs et al., 2007). The tumor inflammatory milieu can be affected by altered p53 status in CAFs which is accompanied by an increased rate of tumor metastasis and worse prognosis. Mechanically, p53 dysfunction in CAFs can promote tumor invasion and malignancy through upregulation of chemokines and cytokines, including CXCL12 and SDF-1 (**Figure 1**; Moskovits et al., 2006; Addadi et al., 2010). Surprisingly, Arandkar et al. (2018) found that non-mutated CAF p53 is functionally distinct from normal fibroblast p53. p53 in lung-derived CAFs is usually hypophosphorylated and is able to modify the transcriptional program, affect the CAF secretome, and promot cancer cell migration and invasion. Overall, tumor progression may require functionally altered p53 in CAFs, and it can be speculated that agents capable of "re-educating" p53 in cancer-associated stromal cells may be able to provide clues for cancer therapy (Arandkar et al., 2018).

Extracellular Matrix Remodeling

One of the most important components in TME is the extracellular matrix (ECM), which are comprised of various macromolecules that regulate cellular functions in tumors. Tumor cells manipulate the arrangement and orientation of ECM to enhance tumor progression and create a positive tumourigenic feedback loop (Cox and Erler, 2011). Previous studies have demonstrated that p53 expression and nuclear localization are modulated by ECM signals (Li et al., 2003). In recent years, the role of p53 in regulating ECM has been verified especially in hypoxic contexts (Petrova et al., 2018). In hypoxic tumor environments, the activation of transcription factors hypoxia inducible factors (HIF) results in the expression of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), which directly participate in the rearrangement of ECM. It has recently been reported that the formation of HIF-1/GOF mutp53 complex in hypoxic cancer cells promotes the transcription of protumourigenic genes and codifys the components of ECM (**Figure 1**; Amelio et al., 2018). p53 can also negatively regulate extracellular matrix metalloproteinase inducer (EMMPRIN), a transmembrane glycoprotein known to promote metastasis and invasion of tumor by enhancing the production of several matrix metalloproteinases (MMPs) (**Figure 1**; Zhu et al., 2009). All these findings underscore the importance that restoring the function of p53 in the ECM may help in the development of anti-cancer therapies.

Immune Cells

Immune cells are important cellular compartments in TME that are heterogeneous across tumor types and are associated with cancer progression and prognosis (Angell and Galon, 2013;

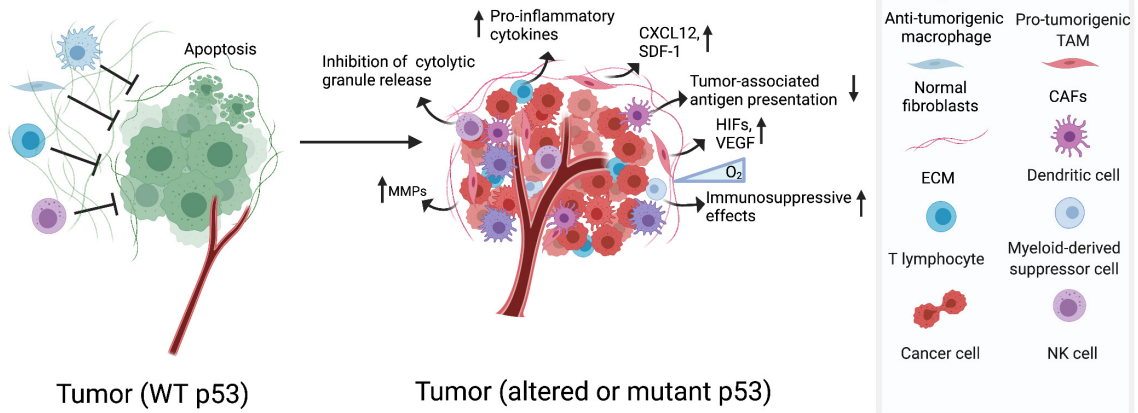

FIGURE 1 | Dysfunction of p53 shapes its immunogenic niche. The TME contains cellular and molecular components that shape immunological landscape of growing tumors. Altered or mutant p53 functions in CAFs increases expression of chemokines and cytokines, which drives tumor invasion and malignancy. In hypoxic tumor environments, GOF mutp53 cooperate with HIF-1 promoting expression of a subset of protumourigenic genes which participate in the rearrangement of ECM. In myeloid cells, p53 deficiency helps to accelerate tumourigenesis. Deletion of p53 in T cells increases the expression of pro-inflammatory cytokines which could help to enhance tumor progression.

Sun et al., 2015; Mlecnik et al., 2016a,b). Productive antitumour immunity largely relies on the tumor-reactive T cells. However, the cytotoxicity of T cells are frequently frustrated in the TME, where the cross-talk between MDSC, macrophages, DC and Treg amplifies the anti-tumor immune effects (Ostrand-Rosenberg et al., 2012).

The function of immune cells can also be regulated by p53. Previous studies have reported that $p53^{-/-}$ mice show more susceptibility to inflammation and auto-immunity which favors tumor establishment and progression (Okuda et al., 2003; Zheng et al., 2005; Guo et al., 2017). And the function of p53 in various immune cells has also been dissected. For instance, p53 deficiency in myeloid lineage accelerates tumourigenesis in an intestinal cancer model, and activation of p53 attenuates the inflammatory response and resists tumor development (**Figure 1**; Guo et al., 2013; He et al., 2015). Furthermore, deficiency of p53 in T cells spontaneously develops inflammatory lesions and autoimmunity, which may help promote tumor development (Zhang et al., 2011).

However, p53 also has a role in regulating the polarization of $CD4^+$ T cells by enhancing the transcription of Foxp3, a master regulator of Tregs, which predicts that the loss of this role of p53 could enhance antitumour immunity (Kawashima et al., 2013). Moreover, deletion of p53 in cytolytic T cells exhibits enhanced glycolytic commitment and reduces murine melanoma (Banerjee et al., 2016). The concept that p53 deletion in T cells enhances antitumour immunity is interesting. However, it may be influenced by other stromal compartments, as p53-deficient mice have substantially faster subcutaneous tumor growth and more regulatory T cells compared to wild-type controls (Guo et al., 2013).

More recently, studies from Dr. Weiping Zou's team reveal that targeting p53–MDM2 interactions augments MDM2 in T cells, thereby stabilizing STAT5 and improving T cell-mediated anti-tumor immunity. Interestingly, these effects are independent of the p53 status of the tumor. Therefore, targeting this pathway could be explored to develop and select additional MDM2-targeted drugs independent of tumor p53 status (Zhou et al., 2021).

Together, these results highlight the important role of p53 in maintaining appropriate TME to suppress tumourigenesis and the potential development of new therapeutic approaches by targeting the p53 pathway.

Compelling evidence suggests that effective cancer therapy requires a multifaceted and integrated approach that not only exposes the tumor but also induces strong anti-tumor immunity. However, current approaches have focused on activating or restoring p53 function in cancer cells. As mentioned above, activation of p53 in TME also affects the immune response. Furthermore, local activation of the p53 pathway rather than overall activation may be sufficient to cause tumor death. Therefore, activation of p53 in TME is an exciting strategy for improving antitumour therapy in the future.

p53 FUNCTIONS IN INNATE AND ADAPTIVE IMMUNITY

The Role of p53 in Innate Immunity

As the first line of defense to detect invaders, innate immune cells are engaged in immediate short-term immune operations upon detection of pathogenic threats to attack and engulf the outsider without establishing immunological memory. The activation of innate immunity is initiated by the stimulation of cell-surface or intracellular pattern recognition receptors (PRRs), including retinoic-acid- inducible gene I (RIG-I)-like receptors

(RLRs), stimulator of IFN genes (STING) protein, and Toll-like receptors (TLRs) (Kawai and Akira, 2010; Trinchieri, 2010; Burdette et al., 2011). The role of p53 in antiviral response has been well reviewed. Here, we discuss how p53 functions in innate immunosurveillance of tumor cells.

The TLRs are membrane glycoproteins and previous studies reveal that p53 transcriptionally regulate several TLRs, constituting a crucial bridge between cellular stresses and TLR-induced innate immune response (Taura et al., 2008; Menendez et al., 2011). Notably, TLR4 has been reported to possess dichotomous role during breast cancer growth, based on the status of p53. TLR4 activation in wtp53 cancer cells leads to the secretion of anti-inflammatory cytokines into microenvironment, resulting in the induction of p21 and cell growth arrest. By contrast, TLR4 activation in mutp53 cells increases secretion of progrowth cytokines such as CXCL1 and CD154. Furthermore, the influence of p53 status on TLR4 activity may extend across cancer types, suggesting that the connection between TLR4 and p53 may provide a therapeutic clue for specifically targeting mutp53 tumors (Haricharan and Brown, 2015).

The cGAS-STING pathway also plays essential role in anti-tumor immunity *in vivo via* up-regulation of type I IFNs (Ablasser and Chen, 2019). More recently, Ghosh et al. (2021) reported that GOF activity of mutp53 can antagonize the STING/TBK1/IRF3 pathway. Mutp53, but not wtp53, binds to TANK-binding protein kinase 1 (TBK1), preventing the formation of the STING-TBK1-IRF3 trimeric complex, which is required for cytokine production and ultimately leads to the onset of immune evasion (**Figure 2**). This finding may provide a key clue to therapeutic approaches aimed at restoring TBK1 function to reactivate immunosurveillance in mutp53-expressing tumors.

Besides myeloid cells, NKG2D-mediated NK cells are also regulated by p53. Restoration of p53 upregulates cell surface expression of ULBP1 and ULBP2 (the NKG2D ligands) that enhance NK cell-mediated cytotoxicity (Textor et al., 2011). However, activation of p53 by Nutlin-3a reduces the expression of ULBP2 in melanoma cells due to the induction of miR-34a/c (Heinemann et al., 2012; **Figure 2**). Thus, it appears that the effect of p53 activation on innate immune regulation is governed by the conditions of its induction.

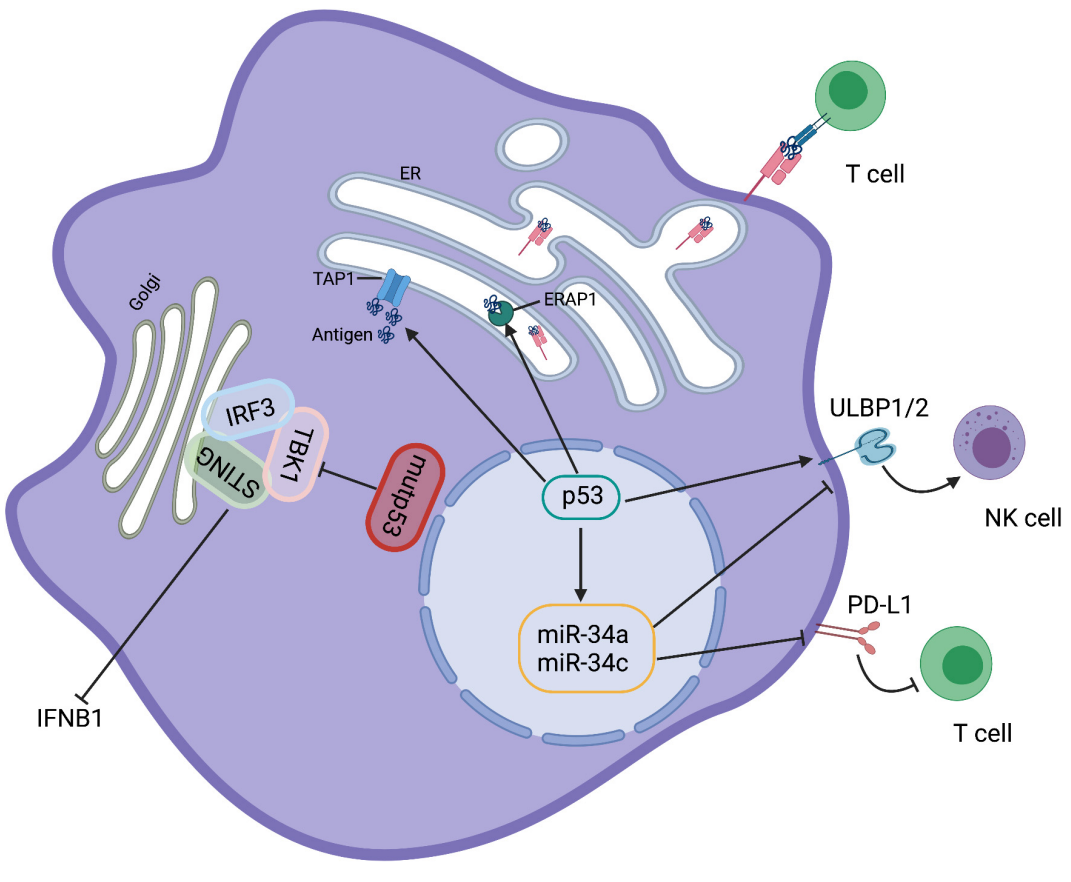

FIGURE 2 | p53 regulates immune responses. Mutp53 binds to TBK1 preventing the formation of STING-TBK1-IRF3 trimeric complexes and rendering immune evasion. p53 upregulation ULBP1 and ULBP2, the NKG2G ligands, to enhance NK cell-mediate cytotoxicity. However, activation of p53 in melanoma cells increases the expression level of miR34, a ULBP2 inhibitor that reduces the recognition of tumor cells by NK cells. Moreover, p53 and miR-34a can cooperate to regulate tumor immune evasion *via* PD-L1. In addition, activation of p53 is able to promote the transport and expression of MHC-I by upregulating TAP1 and ERAP1.

The Role of p53 in Adaptive Immunity

The development of effective immunotherapies for oncology patients is now becoming a clinical reality. Notably, the interaction between T cells and DCs is developing as one of the key targets for immunotherapy. As an important sensor to activate adaptive immune responses, p53-mediated activation of innate immune cells, particularly DC, is expected to promote adaptive immunity. Although the direct effect of p53 on the function of DCs has not been clarified, many results suggest that p53 activation is necessary for DC function. Treatment with Nutlin 3, an MDM2 inhibitor that activates wild-type p53, has been reported to increase the ability of DCs to stimulate T-cell proliferation, suggesting that p53 is involved in the activation of DCs (Gasparini et al., 2012). It has also been shown that the induction of p53 promotes peptide processing and MHC-I expression on the cell surface (**Figure 2**; Zhu et al., 1999; Wang et al., 2013). Therefore, it can be speculated that the enhancement of DC function by p53 may further improve the induction of cytotoxic $CD8^+$ T cells, and the direct role of p53 in DC antigen presentation requires further exploration.

Cancer cells normally upregulate immune checkpoint molecules such as programmed cell death 1 ligand 1 (PD-L1) and cytotoxic T lymphocyte antigen 4 (CTLA4), which are important for T cell tolerance to evade immune attack (Le Mercier et al., 2015; Sharma and Allison, 2015; Baumeister et al., 2016). Links between p53 and immune checkpoints have recently been uncovered. IFN-γ-induced upregulation of PD-L1 expression in melanoma is dependent on p53 (Thiem et al., 2019). Moreover, a number of microRNAs (miRs), which are targets for p53, also play an important role in adaptive and innate immunity. For example, as a transcriptional target of p53, miR-34a inhibits the expression of PD-L1, and dysfunction of p53 increases PD-L1 expression, thereby suppressing T-cell function (**Figure 2**). This result indicates that p53 and miR-34a cooperate to regulate tumor immune evasion *via* PD-L1 (Cortez et al., 2016). Consistent with this, tumor cells carrying p53 dysfunction are usually accompanied with increased expression of PD-L1, which may help to identify patients who respond to immune checkpoint inhibitors against PD-L1 (Cha et al., 2016; Cortez et al., 2016; Biton et al., 2018; Blagih et al., 2020a).

p53 AND DEAD CELL CLEARANCE

During the resolution of injury and infection, normal cell turnover and clearance is an important process in preventing autoimmunity and triggering immune recognition of antigens by dying cells (Green et al., 2009). Failure to sustain efficient clearance is the key contributor to foster disease such as cancer and chronic inflammatory (Elliott and Ravichandran, 2010; Nagata et al., 2010; Fuchs and Steller, 2011; Arandjelovic and Ravichandran, 2015). In normal immune system, phagocytosis of dying cells can induce some degree of immune tolerance to prevent self-antigen recognition. p53 is well-documented as an important regulator of apoptosis, and the role of p53 involved in post-apoptosis has been recently identified. The immune checkpoint regulator DD1α has been reported to be a direct transcriptional target of p53. p53-induced expression of DD1α enhances clearance of apoptotic cells by promoting phagocytosis of macrophages, suggesting that p53 provides protection against inflammatory diseases caused by apoptotic cell accumulation (Yoon et al., 2015). Interactions between macrophage DD1α and T cell DD1α were also observed, making them susceptible to immunosuppression (Zitvogel and Kroemer, 2015). Therefore, this association warrants further preclinical characterization as a potential therapeutic target.

POTENTIAL OF p53 IN IMMUNOTHERAPY

As mentioned above, the regulation of p53 in the tumor immune response exhibits a yin-yang balance. On the one hand, p53 counteracts pro-inflammatory factors, such as NF-κB and STAT3, to promote tissue homeostasis and avoid excessive immune responses. On the other hand, p53 contributes to the recognition of non-self antigens and thus activates anti-tumor immunity through multiple pathways. All these p53 features will allow us to develop more effective tumor therapies in combination with current immunotherapies.

Mutant p53 as a Tumor Antigen

Cancer cells are always accompanied by unstable genetic changes and produce new antigens that distinguish cancer cells from normal cells. The accumulation of p53 hotspot mutations in cancer has been considered as immunologically active neoantigens for immunotherapy. However, progress in this field has been limited by the lack of efficiency of recognition of mutant p53 antigens in cells (Yen et al., 2000; Nijman et al., 2005; Lane et al., 2011). A recent clinical trial in metastatic ovarian cancer showed that p53 hotspot mutations (G245S and Y220C) cause infiltration of mutation-reactive T cells into ovarian cancer metastases (Deniger et al., 2018). A subsequent analysis of 140 patients with multiple types of epithelium confirmed this observation (Malekzadeh et al., 2019). p53 neoantigen-specific HLA-restricted elements and TCRs were found in thirty percent of patients carrying p53 hotspot mutations. And TIL and TCR genetically engineered T cells recognize tumor cell lines that endogenously express these p53 neoantigens. These results highlight the potential of p53 mutations as targets for T cell immunization and gene therapy. Furthermore, the increased levels of p53 protein associated with its mutation are associated with the production of anti-p53 autoantibodies, reinforcing the potential role of p53 in regulating tumor antigenicity (Couch et al., 2007; Garziera et al., 2015). Although mutant p53 has shown promise in the field of immunotherapy, induction of a specific anti-tumor response can trigger immune evasion in some cases. Recent studies have demonstrated the use of a broad-acting vaccine produced by a dendritic cell/tumor cell fusion that can potentially prevent adaptive immune evasion (Humar et al., 2014).

p53 and Immune Checkpoint Inhibitor Therapy

Although significant advances have been made in antitumour immune checkpoint inhibitor (ICI) therapy, only a minority of

cancer patients respond well to immune checkpoint inhibitors (Fares et al., 2019). An effective adaptive immune response requires efficient entry of fully activated cytotoxic T cells into the tumor environment and sufficient tumor-associated antigens that are presented on major histocompatibility complex (MHC) by antigen-presenting cell (APC) (Brown et al., 2018). However, many neoantigen-rich tumors fail to produce a positive immune response in many cancer patients (Stronen et al., 2016). Therefore, amplification of neoantigen libraries remains a promising direction for improving ICI treatment. In recent years, the concept of immunogenic cell death (ICD) has emerged, whereby dying cells stimulate an immune response to antigens released especially from dead cancer cells (Kroemer et al., 2013). Immunochemotherapy has been shown to sensitize tumors to anti-PD1 antibody therapy using clinically relevant mouse models of checkpoint inhibitor resistance (Pfirschke et al., 2016). In addition, Ad-p53 (p53 adenovirus) tumor suppressor immunogene therapy significantly reverse anti-PD-1 resistance in mouse models (Sobol et al., 2017). All these results suggest that chemotherapy-induced p53-dependent apoptosis facilitates the induction of immunogenesis. Indeed, nutlin-3-induced local p53 activation could alter the immune landscape of TME and enhance antitumour immunity by inducing ICD (Guo et al., 2017).

CONCLUSION

As a tumor suppressor, the cell-autonomous function of p53 in suppressing malignant tumors has been extensively studied. More recently, growing evidence suggest a potential link between p53 and immune function, and dysfunction of p53 is also associated with inflammatory diseases. Dysfunction of p53 in tumors is shown to regulate not only immune recognition but also affect the stromal compartment, which plays an important role in controlling tumor progression. Thus, as a "guardian of genomic integrity," p53 also functions in response to homeostatic stress, including innate and adaptive immunity as described above. There are still many uncharacterized issues that presumably have a broad impact on immunity and inflammation, which may ultimately lead to tumor development. For instance, how exactly p53 dysregulation affects the immune response to various external or internal stimuli, and what is the role of p53 in immune cell development. Moreover, depletion or mutation of p53 is likely to reprogram the microenvironment, especially the extracellular components in tumors, but the molecular regulatory mechanisms involved remain still largely unknown. p53 mutations can promote tumor cell metastasis. How the immune regulation and response are changed during this process, and in particular which immune cells' functions are altered. In addition, the role of p53 in the remote regulation and communication between different tissues or organs will also be a highly anticipated research direction. There is no doubt that, understanding these issues will significantly improve our knowledge of both biologic and pathologic functions of p53, allowing for the development of targeted therapeutic approaches in the future.

AUTHOR CONTRIBUTIONS

DS and PJ conceived the manuscript. DS reviewed the literature, drafted the manuscript, and drew the figures. PJ revised the manuscript. Both authors read and approved the final manuscript.

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REVIEW
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Clinical and Immunological Effects of p53-Targeting Vaccines

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Immunotherapy, including immune checkpoint blockade and chimeric antigen receptor T cells, is one of the most promising approaches to treat cancer. Vaccines have been effective in preventing cancers like liver cancer and cervical cancer with a viral etiology. Instead of preventing disease, therapeutic cancer vaccines mobilize the immune system to attack existing cancer. p53 is dysregulated in the majority of human cancers and is a highly promising target for cancer vaccines. Over twenty clinical trials have targeted p53 in malignant diseases using vaccines. In this work, we review the progress of vaccinations with p53 or its peptides as the antigens and summarize the clinical and immunological effects of p53-targeting vaccines from clinical trials. The delivery platforms include p53 peptides, viral vectors, and dendritic cells pulsed with short peptides or transduced by p53-encoding viruses. These studies shed light on the feasibility, safety, and clinical benefit of p53 vaccination in select groups of patients, implicating that p53-targeting vaccines warrant further investigations in experimental animals and human studies.

Keywords: p53, vaccine, Cancer, immunotherapy, T cell

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INTRODUCTION

TP53 gene encodes the transcription factor p53, one of the most important tumor suppressors. Under physiological conditions, p53 expression is tightly controlled and maintains a low level due to rapid degradation by the ubiquitin-mediated proteolysis. The E3 ubiquitin ligase MDM2 and its structural homolog MDMX (also known as MDM4) are the best known negative regulators of p53 (Manfredi, 2021). MDM2 polyubiquitylates p53 and results in proteasome-mediated degradation and monoubiquitylates p53 lead to export p53 out of the nucleus (Wu and Prives, 2018). Furthermore, MDM2 direct interacts with p53 to disrupt the transcriptional activity (Wu and Prives, 2018). Finally, MDM2 is a p53 target gene, thus creating an auto-regulatory feedback loop. MDMX has no ubiquitylation activity, but it binds p53 and inactivates it directly or heterodimerizes with MDM2 to aid MDM2 in p53 ubiquitylation (Wade et al., 2010; Karni-Schmidt et al., 2016; Yang et al., 2021). Under cellular stress, p53 becomes activated and stabilized to transcriptionally regulate target genes that are pivotal to various cellular processes, including cell cycle arrest, apoptosis, and DNA repair (Janic et al., 2018; Boutelle and Attardi, 2021).

P53 IN CANCER

Germline or somatic mutation in the *TP53* gene is frequently found in human cancers (Malkin et al., 1990; Hollstein et al., 1991). Missense mutations are the most common mutation type in cancer tissues, leading to mutant p53 accumulation in tumor cells (Petitjean et al., 2007; Mantovani et al.,

2019). Mutations occur throughout the p53 protein but predominantly are located at exons 4–9 that encode the DNA binding domain, including six "hotspot" residues, namely R175, G245, R248, R249, R273, and R282 (Leroy et al., 2014; Bouaoun et al., 2016). Missense *TP53* mutations are classified as contact or structural mutations. Contact mutations, such as R248Q, R273H, and R273C, disrupt p53 DNA binding, resulting in loss of essential protein-DNA contacts. Structural mutations, such as R175H, G245S, Y220C, and R249S, destabilize the p53 structure and reduce its thermostability (Joerger and Fersht, 2007). Most p53 mutants not only lose wild-type (WT) p53 activity (i.e., loss of function [LOF]) but also obtain dominant-negative (DN) functions to antagonize the remaining WT p53 (Nakayama et al., 2020; Tang et al., 2020). Furthermore, many p53 mutants acquire gain of function (GOF) activity (Nakayama et al., 2020; Tang et al., 2020), though this is debated (Boettcher et al., 2019). Thus, mutant *TP53* acts as an oncogene that promotes tumor cells' survival, proliferation, invasion, and metastasis. However, the *TP53* mutation rates differ significantly in anatomical tumor sites (Wang and Sun, 2017). A Pan-Cancer cohort showed *TP53* was the most frequently mutated gene (42% of samples); it is mutated in 95% serous ovarian cancer, but only in 2.2% renal clear cell carcinoma (Kandoth et al., 2013). The incidence of the LOF p53 mutations is associated with increased chemotherapy resistance and lower efficacy of anti-tumor agents (Keshelava et al., 2001). Overexpress WT p53 in tumor cells increase p53 protein level and lead to cell growth arrest or apoptosis (Ramqvist et al., 1993; McIlwrath et al., 1994). Furthermore, mouse models have demonstrated that the significance of p53 as a regulator of tumor suppression and therapy *in vivo* (Iwakuma and Lozano, 2007). p53 upregulates the expression of CDKN1A, BAX, PUMA, and NOXA, resulting in cell-cycle arrest, apoptosis, and senescence *in vivo* (Brady et al., 2011; Li et al., 2012).

p53 also plays a pivotal role in regulating inflammation in cancer through its activities in non-cancer cells. Lowe and colleagues showed that in the presence of chronic liver damage, ablation of a p53-dependent senescence program in hepatic stellate cells enhanced the transformation of adjacent epithelial cells into hepatocellular carcinoma by skewing macrophage polarization towards a tumor-promoting M2-state (Lujambio et al., 2013). Mice with a targeted deletion of p53 in myeloid cells selectively lost the $Ly6c^{+}CD103^{+}$ population and became unresponsive to immunotherapy and immunogenic chemotherapy, supporting that p53 drives differentiation of monocytic precursor cells into dendritic cells and macrophages for cross-presentation of tumor antigens (Sharma et al., 2018). MDM2 promoted T cell-mediated anti-tumor immunity by preventing c-Cbl-mediated STAT5 degradation; targeting the p53-MDM2 interaction with a pharmacological agent (APG-115) augmented MDM2 in T cells, boosted T cell immunity, and synergized with cancer immunotherapy (Zhou et al., 2021).

Multiple p53-targeting therapeutic strategies have been attempted. For tumors with WT p53, the approach is to suppress the interaction between p53 and MDM2/MDMX, inhibit the degradation of WT p53, and maintain the needed levels of p53 in cells, thus promote tumor suppression. For tumors with mutant p53, therapeutic agents are developed to reactivate mutant p53 or promote its degradation (Chen et al., 2021). Targeting the p53 signaling pathway has been extensively reviewed by a number of investigators (Hernández Borrero and El-Deiry, 2021; Huang, 2021; Liu et al., 2021; Salomao et al., 2021). At least theoretically, therapeutic restoration of inactivated tumor suppressors is more challenging than inhibiting an oncogenic target. Indeed, no therapy has successfully reactivated a mutated tumor suppressor in a clinical setting. Furthermore, p53 is an intracellular protein, making it inaccessible to antibodies.

CANCER IMMUNOTHERAPY

Cancer immunotherapy manipulates the immune system to recognize and destroy cancer cells. Therapeutic cancer vaccines are an exciting development in cancer immunotherapy by eliciting specific immune responses to tumor antigens. $CD8^{+}$ cytotoxic T lymphocytes (CTLs) are preferred effector cells for anti-tumor immune responses (Hossain et al., 2021). $CD8^{+}$ CTLs act as the key player in mediating tumor suppression through recognition of tumor-specific or associated antigens. T lymphocytes recognize antigens presented by antigen-presenting cells (APCs) in a major histocompatibility-restricted manner. Dendritic cells (DCs), discovered and characterized by Steinman and Cohn in 1973, are the most efficient APCs (Steinman and Cohn, 1973; Steinman and Cohn, 1974; Steinman et al., 1975). DCs participate in a variety of immunological processes, including initiating immune responses and sustaining effective T-cell-mediated anti-tumor immune responses (Marciscano and Anandasabapathy, 2021). DCs are classified into immature and mature according to their developmental stage. When immature DCs recognize, uptake, and cross-present the antigens released by tumor cells, they shift to secondary lymphoid organs, where they activate $CD4^{+}$ T cells or $CD8^{+}$ T cells to trigger specific CTLs responses against target cells (Wang et al., 2020a).

VACCINES TARGETING P53

Both WT and mutant p53 epitopes can be presented on the cell surface in the context of MHC I molecules by APCs for $CD8^{+}$ T cell recognition (Houbiers et al., 1993). CTLs recognizing the WT $p53_{25–35}$, $p53_{110–124}$, $p53_{108–122}$, $p53_{149–157}$, and $p53_{264–272}$ epitopes have been reported, and many are used for developing potentially broadly applicable cancer vaccines (Chikamatsu et al., 1999; Chikamatsu et al., 2003; Rojas et al., 2005; Ito et al., 2006). $p53_{110–124}$ specific $CD4^{+}$ T cells promote the generation and function of tumor-specific $CD8^{+}$ CTLs (Chikamatsu et al., 2003). Short peptides from a mouse mutant p53 are recognized by $CD4^{+}$ and $CD8^{+}$ T cells, and vaccination with a mutant peptide emulsified in incomplete Freund's adjuvant leads to tumor inhibition (Noguchi et al., 1994; Theobald et al., 1995). p53 mutations are associated with overexpression of mutant p53 in cancer cells, which may lead to the abnormal presentation of p53

TABLE 1 | Clinical trials with p53-targeting vaccines in human Cancers.

Author	Year	Phase	Vaccine platform	Antigen[a,b]	Disease	Patient no	Disease state	Previous treatment	Immunizations (x)
Kuball	2002	Pilot study	Recombinant adenovirus	WT FL p53	Urogenital, lung cancer, malignant schwannoma	6	Advanced disease	Unknown	4
Menon	2003	I/II	Recombinant canarypox virus	WT FL p53	Colorectal cancer	16	Metastatic disease	Chemotherapy/ radiation therapy/other	3
Svane	2004	I	Short peptide-pulsed DC	WT p53 peptide	HLA-A2$^+$ breast cancer	6	Metastatic disease	Chemotherapy/ radiotherapy/ endocrine	10
Lomas	2004	I	Short peptides plus GM-CSF	Short peptides derived from human anti-p53 (WT denatured) antibodies	Breast, colorectal, non-small-cell lung, renal, prostate, head- and neck, hemangiopericytoma, esophageal cancer	14	NED/metastatic /recurrent disease	Yes	4
Antonia	2006	I/II	Recombinant adenovirus-transduced DC	WT FL p53	Small cell lung cancer	29	Extensive/ recurrent disease	Chemotherapy	3 or 6
Herrin	2007	II	Short peptide-pulsed DC	Short WT p53 peptide	HLA-A2$^+$ ovarian cancer	21	Advanced/ recurrent disease	Yes	>3
Svane	2007	II	Short peptide-pulsed DC	3 WT p53 peptides + 3 mutant p53 peptides (to enhance HLA-A2 binding	HLA-A2$^+$ breast cancer	26	Metastatic	Regimens/ endocrine	10
Leffers	2009	II	Long peptide	10 long peptides covering WT p53 (70–248)	Ovarian cancer	20	Recurrent disease	Surgery/ chemotherapy	4
Speetjens	2009	I/II	Long peptide	10 long peptides covering WT p53 (70–248)	Colorectal cancer	10	Metastatic disease	Surgery/ chemotherapy	2
Yoo	2009	II	Recombinant adenovirus	WT FL p53	Squamous Cell Carcinoma	13	Advanced disease	No	2
Trepiakas	2010	I/II	Short peptide-pulsed DC	Short peptides for p53 (mutated to enhance HLA-A2 binding), survivin, and telomerase Long peptide	Melanoma	46	Metastatic disease	Chemotherapy	1–29
Rahma	2012	II	Short peptide pulsed-DC	Short WT p53 peptide	HLA-A2$^+$ ovarian cancer	21	Advanced/ recurrent disease	Yes	4
Vermeij	2012	II	Long peptide	10 long peptides covering WT p53 (70–248)	Ovarian cancer	12	Recurrent disease	Chemotherapy	4
Iclozan	2013	II	Recombinant adenovirus-transduced DC	WT FL p53	Small cell lung cancer	56	Advanced disease	Chemotherapy/ radiotherapy	3
Zeestraten	2013	I/II	Long peptide plus IFN-α	10 long peptides covering WT p53 (70–248)	Colorectal cancer	11	Metastatic disease	Surgery/ chemotherapy/ radiotherapy	2
Hardwick	2014	I	Recombinant vaccinia Ankara virus	WT FL p53	Pancreatic cancer, colon cancer	12	Unresectable and chemotherapy-resistant disease	Chemotherapy/ radiotherapy	3
Schuler	2014	I	Short peptide-pulsed DC	Short WT p53 peptides (mutated to	HLA-A2$^+$ HNSCC	16	Advanced disease	Surgery/ chemotherapy	3

(Continued on following page)

TABLE 1 | (*Continued*) Clinical trials with p53-targeting vaccines in human Cancers.

Author	Year	Phase	Vaccine platform	Antigen[a,b]	Disease	Patient no	Disease state	Previous treatment	Immunizations (x)
				enhance HLA-A2 binding)					
Dijkgraaf	2015	I/II	Long peptide	10 long peptides covering WT p53 (70–248)	Ovarian cancer	8	Platinum-resistant disease	Chemotherapy	2
Hardwick	2018	I	Recombinant vaccinia Ankara virus	WT FL p53	Ovarian cancer	12	Platinum-resistant disease	Chemotherapy	3
Soliman	2018	I/II	Recombinant adenovirus-transduced DC	WT FL p53	Breast, colon, gastric, lung, tongue, ovarian, chondrosarcoma cancer	194	Metastatic disease	Chemotherapy	4
Chiappori	2019	II	Recombinant adenovirus-transduced DC	WT FL p53	Small cell lung cancer	78	Recurrent disease	Chemotherapy	3
Chung	2019	I	Recombinant vaccinia Ankara virus	WT FL p53	Breast, pancreatic, hepatocellular, or head and neck cancer	11	Recurrent disease	Chemotherapy	3

[a]*WT, wild-type.*
[b]*FL, full-length.*

peptides by APCs. $p53_{264–272}$ or $p53_{149–157}$ tetramer$^+$ CD8$^+$ CTLs have been detected in the circulation of head and neck squamous cell carcinomas (HNSCC) patients and negatively correlated with p53 expression in tumor tissues and tumor stage (Albers et al., 2018). These reports suggest that p53-specific CD8$^+$ CTLs could eliminate tumor cells, as the immune system has the ability to recognize the p53 epitopes represented on the surface of cancer cells and APCs. Based on this evidence, a growing number of clinical trials targeting p53 have been conducted (Vermeij et al., 2011; DeLeo and Appella, 2020). This work comprehensively reviews these clinical trials with therapeutic vaccines against p53. **Table 1** contains the clinical trial enrollment information such as vaccine platform, antigen type, and cancer type, and **Table 2** provides the information on induced immune and clinical responses in cancer patients.

Peptide Vaccines

Lomas et al. conducted a phase I trial with up to four doses of a pool of eight short peptides derived from the complementarity determining regions of human anti-p53 antibodies (Lomas et al., 2004). In this trial, 14 patients with solid tumors were enrolled, and six received all four idiotypic vaccinations. The serum anti-vaccine antibodies were mainly IgG. One patient had increased titers of anti-p53 antibodies. Two patients showed responses in the thymidine proliferation assay to immunized peptides. In contrast to the proliferation assays, no patients had vaccine-specific, IFN-γ-secreting T cells as assessed by the enzyme-linked immune absorbent spot (ELISpot) assay (Lomas et al., 2004).

Leffers et al. conducted a phase II trial with a p53 synthetic long peptide (p53-SLP) vaccine in 20 ovarian cancer patients (Leffers et al., 2009). The p53-SLP vaccine contains 10 25–30 amino acid long peptides covering WT p53 from position 70–248. Before immunization, eight of 20 patients had p53 auto-antibodies associated with p53 expression in primary tumors. After immunization, nine presented p53-autoantibodies. Before immunization, responses against peptides included in the vaccine were present in three patients. After completing the immunization scheme, all patients had detected vaccine-induced IFN-γ producing p53-specific T-cells. These p53-specific IFN-γ T cells were CD4$^+$CD8$^-$. Two of the total 20 patients had stable disease as evaluated by CA-125 and computerized tomography (CT) and they had vaccine-induced p53-specific responses. Eighteen of 20 patients had clinical, biochemical, and/or radiographic evidence of progressive disease (Leffers et al., 2009). The authors concluded that the p53-SLP vaccine does not affect responses to secondary chemotherapy or survival and that p53-specific T cells do survive chemotherapy (Leffers et al., 2012).

The p53-SLP vaccine combined with cyclophosphamide therapy is evaluated by Vermeij et al. for treating patients with recurrent ovarian cancer in a single-arm phase II study (Vermeij et al., 2012). Twelve patients were administered four doses of the p53-SLP vaccine at a 3 week interval. Two days before each immunization, patients were treated with cyclophosphamide infusion. After four immunizations, seven of eight evaluable patients displayed vaccine-induced IFN-γ-producing p53-specific T cells, and five produced both T-helper 1 and T-helper-2 cytokines. The p53-SLP vaccine and cyclophosphamide combination therapy had no effect on T_{reg} cells. Two patients had stable disease as evaluated by serum CA-125 measurement and CT scan, and vaccine-induced p53-specific responses were present in both patients (Vermeij et al., 2012).

Speetjens et al. conducted a phase I/II trial with two doses of the p53-SLP vaccine in 10 metastatic colorectal cancer patients (Speetjens et al., 2009). Six of nine vaccinated patients with p53-SLP had p53-specific immune response detected by IFN-γ ELISpot. Furthermore, two showed detectable p53 specific CD3$^+$CD4$^+$CD137$^+$ cell responses, none had

TABLE 2 | Immune and clinical response p53-targeting vaccines.

Author	Year	Humoral response[a]	ELISpot	CD137 assay	p53-specific proliferation[b]	Treg frequency decrease	MDSC frequency decrease	Immunohistochemistry[c]	Clinical response[d,e]	Adverse events (Grade)
Kuball	2002	0/6	0/6	Not analyzed	Not analyzed	Not analyzed	Not analyzed	3/6 positive	4/6 SD, 2/6 PD	1
Menon	2003	Pre 7/15 post 10/15	Not analyzed	Not analyzed	Not analyzed	Not analyzed	Not analyzed	Not analyzed	1/16 SD, 15/16 PD	1/2
Svane	2004	Not analyzed	4/6 PR	Not analyzed	Not analyzed	Not analyzed	Not analyzed	3/6 positive	2/6 SD, 2/6 PD, 2/6 MR/UR	1/2
Lomas	2004	Pre 0/6 post 1/6	0/6 PR	Not analyzed	2/6 VIR	Not analyzed	Not analyzed	14/14 positive	Not analyzed	1/2
Antonia	2006	Pre 10/22 post 10/22	16/28 PR	Not analyzed	Not analyzed	No	Not analyzed	Not analyzed	1/29 PR, 7/29 SD, 21/29 PD	1/2
Herrin	2007	Not analyzed	14/20 PR	Not analyzed	Not analyzed	Not analyzed	Not analyzed	Not analyzed	Not analyzed	1–4
Svane	2007	Not analyzed	8/22 PR	Not analyzed	Not analyzed	Not analyzed	Not analyzed	11/26 positive	8/19 SD, 11/19 PD	1/2
Leffers	2009	Pre 8/20 post 9/20	18/18 PR	Not analyzed	14/17 PR	Not analyzed	Not analyzed	9/20 positive	2 SD, 18 PD	1/2
Speetjens	2009	Not analyzed	6/9 PR	2/10 $CD4^+$ PR, 0/10 $CD8^+$ PR	7/10 VIR	No	Not analyzed	7/10 positive	5/10 NED, 5/10 RD	1/2
Yoo	2009	Not analyzed	Not analyzed	Not analyzed	Not analyzed	Not analyzed	Not analyzed	Not analyzed	3/13 RD	1–4
Trepiakas	2010	Not analyzed	1/4 PR	Not analyzed	Not analyzed	No	Not analyzed	Not analyzed	11/36 SD	1/2
Rahma	2012	Not analyzed	14/20 PR	Not analyzed	Not analyzed	No	Not analyzed	Not analyzed	4/20 NED, 16/20 RD	1–4
Vermeij	2012	Not analyzed	7/8 PR	Not analyzed	5/8 VIR	No	Not analyzed	5/12 positive	2/10 SD, 8/10 PD	1/2
Iclozan	2013	Not analyzed	3/15 PR, 5/12 PR	Not analyzed	Not analyzed	No	No	Not analyzed	Not analyzed	Not reported
Zeestraten	2013	Pre 7/8 post 7/8	11/11 PR	Not analyzed	4/9 VIR	Not analyzed	Not analyzed	8/11 positive	Not analyzed	1/2
Hardwick	2014	Pre 0/5 post 5/5	6/6 PR	8/12 $CD4^+$ PR, 10/12 $CD8^+$ PR	Not analyzed	No	5/9	Not analyzed	Not analyzed	1/2
Schuler	2014	Not analyzed	4/16 PR, 11/16 PR (Tetramers)	Not analyzed	Not analyzed	12/15	Not analyzed	8/16 positive	13/16 NED	1/2
Dijkgraaf	2015	Not analyzed	8/8 PR	Not analyzed	0/8 VIR	No	No	Not analyzed	3/6 PD, 1/6 SD, 2/6 PR	1–4
Hardwick	2018	Not analyzed	Not analyzed	5/11 $CD4^+$ PR, 6/11 $CD8^+$ PR	Not analyzed	7/11	6/11	Not analyzed	3/11 SD, 1/6 PR	1–4
Soliman	2018	Not analyzed	7/23 PR	Not analyzed	Not analyzed	Not analyzed	Not analyzed	44/94 positive	4/39 SD	1–5
Chiappori	2019	Not analyzed	13/38 PR	Not analyzed	Not analyzed	Not analyzed	Not analyzed	Not analyzed	2/61 NED, 13/61 SD, 1/61 PR, 35/61 PD	1–3
Chung	2019	Not analyzed	Not analyzed	2/11 $CD4^+$ PR, 2/11 $CD8^+$ PR	Not analyzed	Not analyzed	Not analyzed	Not analyzed	3/11 SD, 6/11 RD	1–4

[a]*Pre- and post-immunization levels of anti-p53-specific antibodies.*
[b]*p53-specific T-lymphocytes induced by immunizations. PR, positive response; VIR, vaccine-induced response.*
[c]*p53-staining of primary tumor samples.*
[d]*SD, stable disease; PD, progressive disease; MR, mixed response, UR, unconfirmed regression; PR, partial response; RD, recurrent disease; NED, no evidence of disease.*
[e]*All according to Response Evaluation Criteria in Solid Tumors.*

$CD3^+CD8^+CD137^+$ cell responses. One patient showed a p53-specific proliferative response before vaccination; seven of 10 patients displayed vaccine-induced p53-specific reactivity after vaccination. Vaccination has no effects on the induction of p53-specific T_{reg} cells. After vaccination, five had no evidence of disease, five showed recurrent disease (Speetjens et al., 2009).

In an ensuing phase I/II clinical trial, the combination of interferon IFN-α and p53-SLP was evaluated in 11 colorectal cancer patients (Zeestraten et al., 2013). The patients were treated with metastasectomy, chemotherapy, and/or radiofrequency ablation (RFA) for disease metastasis. p53-specific IgG antibody responses were detected in seven of eight patients who had serum samples from pre- and post-vaccination. In all patients, p53-SLP vaccination combined with IFN-α treatment-induced p53-specific T-cell responses. The toxicity of the combination was limited to Grade 1 or 2. After the two vaccinations, four of nine patients showed vaccine-induced proliferative responses (Zeestraten et al., 2013).

A phase I/II trial combining gemcitabine, IFN-α and the p53-SLP vaccine was conducted in patients with platinum-resistant ovarian cancer by Dijkgraaf et al. (2015). Patients were sequentially treated in three groups: the first three patients received gemcitabine alone, the following six patients received gemcitabine and IFN-α and the remaining six received gemcitabine, IFN-α, and additionally p53 SLP vaccine. Patients who received gemcitabine/IFN-α/p53 SLP treatment showed profound T-cell activation and increases in activated T-cell/T_{reg} cell ratios. All p53 SLP vaccinated patients showed detectable p53-specific T-cell responses (Dijkgraaf et al., 2015). Eleven Grade 3/4 adverse events were observed, most likely due to chemotherapy and/or IFN-α (Dijkgraaf et al., 2015).

From the above studies, we can conclude that the p53-SLP vaccine is safe and capable to induce p53-specific T-cell responses in patients treated for multiple cancers, yet improved survival is yet to come. Most studies are underpowered to demonstrate efficacy in the specific cancer population.

Recombinant Viral Vaccines

Recombinant viral vaccines aim to use a live virus or attenuated virus to induce an immune response against the viral-encoded antigen (Nascimento and Leite, 2012). There are a number of viral platforms in vaccinations for many pathogens that have thwarted efforts towards control using conventional vaccine approaches (Ewer et al., 2016; Nasar et al., 2017), many of which are used to target p53 in cancer.

In a pilot clinical trial, six advanced-stage cancer patients were immunized with four doses of rAd/hup53 particles (Kuball et al., 2002). rAd/hup53 is a recombinant replication-defective adenoviral vector encoding human full-length WT p53, and it is capable of priming A2.1-restricted and hup53 epitope-specific CTLs *in vivo* but unable to induce p53-specific antibodies. After vaccinations, adenoviral backbone induced $CD4^+$ T cells and $CD8^+$ T cells in six and two patients, respectively. The treatment was well tolerated, yet no evidence for objective tumor responses was observed (Kuball et al., 2002).

The canarypox virus (ALVAC) is a well-characterized viral vector capable of infecting without replicating in mammalian cells. A phase I/II study was performed on 16 colorectal cancer patients with three intravenously injections of increasing dose of ALVAC encoding the human WT p53 gene (ALVAC-p53) at 3 week intervals (Menon et al., 2003). All patients had metastatic disease of p53-overexpressing colorectal cancer. Fever was the only vaccination-related adverse event. Before the vaccination, seven patients had IgG responses against p53; after vaccination, IgG responses against p53 were induced in three more patients (Menon et al., 2003). Following vaccinations, only one patient showed stable disease, while others showed progressive disease (Menon et al., 2003). No anaphylactic reaction or unwanted autoimmune reactions were observed (Menon et al., 2003).

Modified vaccinia virus Ankara (MVA) is a highly attenuated cytopathic strain replication-competent virus, a well-established vaccinia virus that the Food and Drug Administration has approved as a smallpox vaccine. In a phase I trial of p53MVA (an MVA virus carrying the WT p53 gene), 12 patients with refractory pancreatic and colon cancer were treated with three increasing doses of p53MVA every 3 weeks (Hardwick et al., 2014). Activation-induced CD137 expression is a common marker for antigen-triggered T cell responses. In this study, four patients had $CD137^+CD4^+$ T cells, and 10 had $CD137^+CD8^+$ T cells upon stimulation with the p53 peptide library. An MVA antibody neutralization assay showed that all patients had a low anti-MVA response before vaccination, whereas vaccination increased T cell reactivity and neutralizing activity against MVA. Patients with lower frequencies of $PD1^+$ $CD8^+$ T cells had greater p53-reactive $CD8^+$ T cells after immunization, and antibody blockade of PD-1 *in vitro* increased the p53 immune responses (Hardwick et al., 2014). This first-in-human single-agent trial showed the p53MVA vaccine is well tolerated and immunogenic, but it showed no significant clinical responses.

Hardwick et al. conducted a dose de-escalating phase I trial of p53MVA vaccine in combination with the gemcitabine chemotherapy (Hardwick et al., 2018). Twelve patients with platinum-resistant ovarian cancer were enrolled in this trial and treated with gemcitabine before three p53MVA vaccinations. Five patients had $CD137^+$ $CD4^+$ T cells, and six had $CD137^+$ $CD8^+$ T cells after vaccination. In 11 patients evaluated for toxicity of the p53MVA/gemcitabine combination therapy, clinical outcome, and immunologic response, none had complete responses, three had stable disease, and one had a partial response on the second post-therapy CT scan (Hardwick et al., 2018).

Chung et al. conducted a phase I trial with the combination of p53MVA and pembrolizumab (anti-PD-1) to treat patients with advanced solid tumors (Chung et al., 2019). Eleven patients with advanced breast, pancreatic, hepatocellular, or head and neck cancer received up to three dose vaccines combined with pembrolizumab at a 3-week interval. They observed clinical responses in three patients who maintained stable disease for up to 49 weeks. Two of them showed increased p53 specific $CD137^+CD4^+$ and $CD137^+CD8^+$ T cells and upregulated multiple immune response genes (Chung et al., 2019).

Advexin (INGN 201, Ad5CMV-p53) is a replication-impaired adenoviral vector that carries the p53 gene under the

cytomegalovirus (CMV) promoter and is a well-tolerated and efficacious treatment, both as a monotherapy and in combination with radiation and/or chemotherapy agents (Zhang et al., 1994). Yoo et al. conducted a phase II trial of surgery with perioperative INGN 201 gene therapy. Thirteen patients with advanced, resectable squamous cell carcinoma of the oral cavity and oropharynx were treated with INGN 201 along with surgery and chemoradiotherapy. After surgery, all patients received perioperative INGN 201 injections in the primary tumor bed and the ipsilateral neck. In addition, three patients received injections in the contralateral neck. All but three patients received chemoradiotherapy (Yoo et al., 2009). Of the 10 patients with evaluable data, two experienced Grade 4 adverse events and three died with observed relapses before death (Yoo et al., 2009). Overall, the estimate of 1 year progression-free survival was 92%, yet no definitive conclusion can be made with this small sample size.

DCs Pulsed With p53 Peptides

Schuler et al. conducted a randomized phase I trial with p53 peptide-pulsed DCs in patients with HNSCC (Schuler et al., 2014). Both class I and class II peptides from p53 are used in this study: p53-sequences 149–157 with T150L and 264–272 with F270W are HLA-A2.1+ restricted (p53-I), and p53-sequence 110–124 are DR4+ restricted (p53-II). A T-helper [Th] tetanus toxoid peptide (Tt-II) is used as a control for p53-II. 16 HLA-A2.1$^+$ patients were randomized into three arms: six in arm 1 (DCs with p53-I peptides), four in arm 2 (DCs with p53-I peptides + Tt-II peptide), and six in arm 3 (DCs with p53-I peptides + p53-II peptide). Vaccine-pulsed DCs were delivered to inguinal lymph nodes at the third time point. After vaccination, 12 of 15 patients showed decreased T_{reg} cells, and 13 had no evidence of disease in a median follow-up of 32 months. Eight patients had p53-positive tumors, but there was no difference in disease-free survival between patients with p53-positive versus p53-negative tumors (Schuler et al., 2014). There were no Grade 2–4 adverse events.

Svane et al. conducted a phase I trial of vaccination with p53 peptide-pulsed DCs in patients with advanced breast cancer (Svane et al., 2004). Six HLA-A2-associated p53 short peptides were used (3 WT and three were mutated to enhance HLA-A2 binding), along with and a pan-MHC class II peptide, PADRE. Nine patients received 10 immunizations with p53- and PADRE-peptide–pulsed autologous DCs. Before vaccination, two of them had T-cell reactivity against p53 peptides. After four or six vaccinations, four patients showed increased specific T-cell responses against p53 peptides. In three patients with vaccine-induced reactivity, T-cell responses were declined at late time intervals. Two patients maintained stable disease for more than 6 months (Svane et al., 2004).

A phase II trial of vaccination with the same p53 peptide-pulsed DCs for patients with advanced breast cancer was conducted by Svane et al. (2007). This phase II trial enrolled 26 patients with progressive metastatic breast cancer patients. Eight of 22 evaluated patients had p53-specific CTLs after immunization. p53 was frequently expressed in tumors from patients achieving stable disease. Five of six patients with stable disease expressed p53, whereas only six of 18 with progressive disease. Overall, among 19 patients available for first evaluation after six vaccinations, eight had stable disease, and 11 had progressive disease, supporting an effect of p53-specific vaccination (Svane et al., 2007).

Trepiakas et al. conducted a phase I/II trial with DCs pulsed with multiple tumor peptides from p53, survivin, and telomerase in 46 patients with malignant melanoma (Trepiakas et al., 2010). The p53 peptides (and the PADRE peptide) were the same as above. One out of four patients had increased lysate-specific IFN-γ response as detected by ELISpot, and six of 10 showed detectable antigen-specific T cell response as assessed by MHC multimer assays. After six vaccinations, compared to patients with progressive disease, patients with stable disease displayed significantly lower T_{reg} cells. Thirty-six patients had a clinical response: 11 had stable disease, six had continued stable disease after 16 weeks, and six had continued stable disease after 19 weeks (Trepiakas et al., 2010).

In an ensuing phase II study, metastatic melanoma patients were treated with p53 peptides-pulsed DC vaccination with interleukin-2, metronomic cyclophosphamide, and a Cox-2 inhibitor. The same six p53 peptides and the PADRE peptide were used. Among 28 patients evaluated: 16 had stable disease, and 12 had progressive disease (Ellebaek et al., 2012). The authors concluded that DC vaccination in combination with IL-2, cyclophosphamide, and the Cox-2 inhibitor was safe and tolerable, and a general increase in immune responses was observed upon fourth vaccination; however, a correlation between clinical benefit and a vaccine-induced T-cell response could not be determined (Ellebaek et al., 2012).

Herrin et al. conducted a randomized phase II trial with p53 vaccine to compare subcutaneous direct administration with intravenous peptide-pulsed DCs in high-risk ovarian cancer patients (Herrin et al., 2007). A single WT p53 epitope (264–272) with high HLA-A2.1 affinity was used for vaccination. Twenty-one patients were enrolled in this phase II study. On the subcutaneous arm, nine of 13 patients had an immunologic response. On the intravenous arm, five of seven had an immunologic response. Mean overall survival on the subcutaneous and intravenous arm is 70.4 and 72.9 months, respectively (Herrin et al., 2007).

DCs Transduced With Virus

Antonia et al. tested a cancer vaccine based on adenovirus-transduced DCs (Ad.p53-DC) in a phase I/II study (Antonia et al., 2006). The virus expressed a full-length WT human p53. Twenty-nine patients with late-stage small cell lung cancer (SCLC) enrolled and received three vaccinations every 2 weeks. Ten patients had a detectable level of anti-p53 antibody before vaccination, and only three had a significantly increased anti-p53 antibodies level after immunization. p53-specific T cell responses were detected in 13 of 25 patients who underwent the IFN-γ ELISpot assay (Antonia et al., 2006). Among evaluated patients treated with this vaccine, one achieved a partial response, seven showed stable disease, and 21 developed progressive disease (Antonia et al., 2006).

Forkhead box protein P3 (FOXP3) expressing regulatory T (T_{reg}) cells are a subset of $CD4^+$ T cells with high immunosuppressive activity, which is critical cells for maintaining dominant self-tolerance and immune homeostasis (Togashi et al., 2019). T_{reg} cells exert their immunosuppressive activity through various cellular and humoral mechanisms, including cytotoxic T lymphocyte antigen 4 (CTLA-4)-mediated suppression of APCs, consumption of IL-2, and production of immune inhibitory cytokines and molecules (Spolski et al., 2018; Tekguc et al., 2021). T_{reg} cells can suppress anti-tumor immunity, and T_{reg} cells dysregulation is associated with a poor prognosis in human cancer patients (Wang and Ke, 2011; Saito et al., 2016). There are two types of antigens presented in tumor cells, including non-self-antigens, also known as neoantigens, derived from either oncogenic viral proteins or mutant proteins, and self-antigens, which are generated from highly or aberrantly expressed endogenous proteins. Self-antigens reactive $CD8^+$ T cells exhibit an anergic phenotype owing to suppression by T_{reg} cells, while non-self-specific $CD8^+$ T cells showed resistance to T_{reg} cells mediated suppression in humans (Maeda et al., 2014). Thus, T_{reg} cells exert more effective suppression in immune responses against self-antigens than non-self-antigens. Programmed cell death 1 (PD-1) is a negative regulator of T_{reg} cells as well as effector T cells, suggesting that PD-1 blockade enhances the suppressive function of T_{reg} cells (Kamada et al., 2019; Kumagai et al., 2020). The effects of p53-targeting vaccination on T_{reg} cells were estimated in this Ad.p53-DC phase I/II study (Antonia et al., 2006). Before or after vaccination, there was no significant change of these cells in healthy subjects and patients with SCLC, and no statistically significant link between the presence of these cells in the patients' blood and p53-specific T cell responses (Antonia et al., 2006).

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of cells. MDSCs are pathologically activated myeloid progenitors and immature myeloid cells with potent immunosuppressive activity (Hegde et al., 2021; Veglia et al., 2021). A number of studies have demonstrated that MDSCs are implicated in T cell suppression and are closely associated with poor clinical outcomes in cancer (Wang et al., 2020b; Imazeki et al., 2021). Mouse experiment also demonstrated that MDSC depletion with antibodies or different compounds could substantially improve anti-tumor immune responses to exert anti-tumor effects (Gabrilovich et al., 2012). Iclozan et al. conducted a randomized phase II trial in patients with late-stage SCLC using the Ad.p53-DC vaccine (Iclozan et al., 2013). Fifty-six patients were randomized into one of three arms: 18 to arm A (control), 19 to arm B (the Ad.p53-DC vaccine alone), and 19 to arm C (the Ad. p53-DC vaccine plus all-trans retinoic acid (ATRA). ATRA substantially decreases MDSC. Patients were administrated with the Ad.p53-DC vaccine three times at 2 week intervals. The Ad.p53-DC vaccine alone showed no effect on the frequency of MDSC and T_{reg}, while the p53 vaccine combined with ATRA significantly decreased MDSCs (Iclozan et al., 2013). In addition, three patients in arm B had p53-specific immune response, and five in arm C had detectable p53 response (Iclozan et al., 2013). In a following article, Chiappori reported the clinical results of a randomized-controlled phase II trial of patients with recurrent SCLC with the same three arms (Chiappori et al., 2019). No immune response was detected in arm A (control), three of 15 patients showed positive immune responses in arm B, and 10 had positive immune response in arm C. In arm B, two patients maintained complete response, four had stable disease, 13 had progressive disease, and one had partial response. In arm C, nine patients had stable disease and 22 had progressive disease (Chiappori et al., 2019).

A phase-I/II study of the Ad. p53-DC vaccine in combination with indoximod in metastatic tumors were reported by Soliman et al. (2018). Forty-four patients with p53-positive by immunohistochemistry were enrolled in this trial. Seven of 23 patients had increased $CD8^+$ T cell positive response, and six showed increased $CD8^+CD69^+$ T cells at week 3. During the vaccination period, no objective responses occurred; stable disease was observed in four patients at week 7 (Soliman et al., 2018). Overall, the Ad.p53-DC vaccine is safe and elicits immune responses, yet it fails to improve the overall response to chemotherapy.

CONCLUSION

p53 is mutated in about half of all cancers and has attracted great interest in the development of cancer vaccines. An increasing number of studies on p53 vaccines, either peptide-, virus-, or DC-based for cancer immunotherapy, have been reported. Several vaccines have been tested in multiple clinical trials: p53-SLP, p53MVA, DCs with six p53 peptides and the PADRE peptide, and DCs transduced with Ad.p53. Key findings from these published clinical trials are summarized (**Tables 1**, **2**). First, the p53 vaccines themselves are safe, *albeit* patients may have high-grade adverse events when they have adjunctive chemotherapy. Second, p53 vaccines elicit p53-specific immune responses. Third, current p53 vaccines do not improve patient survival to justify even a phase III trial, let alone approved to treat patients. Finally, current p53 vaccines are largely dependent on WT p53 full-length protein or peptides, which may circumvent the avidity of the CTLs due to self-tolerance (Theobald et al., 1997; Kuball et al., 2002). As vaccination technologies have unprecedented progress and successes during the COVID-19 pandemic, we call for further development of personalized p53-targeting vaccines with the following provocative questions. 1) Should we include antibodies in assays testing the B cell responses in addition to T cell activation? Mutant p53 is known to be released into the circulation of cancer patients (Sobhani et al., 2020). It is unclear whether the elicited antibodies from vaccines targeting mutant p53 in the serum or within the tumors offer therapeutic benefits. At a minimum, these antibodies may attract APCs to the tumor sites where mutant p53 antigens enrich. 2) Should we worry about autoimmune reactions targeting the endogenous WT p53 in physiologic tissues? Current p53 vaccines do not show widespread anaphylactic and autoimmune toxicities. It is notable that the R175H neoantigen is only presented 1.3–2.4 copies per cell in several tumor cell lines carrying the R175H

mutation (Hsiue et al., 2021). It is unlikely that low endogenous WT p53 within normal tissues pose a serious challenge for T cell autoimmune response. 3) Will improved delivery methods help? mRNAs encapsulated in lipid nanoparticles (LNP) elicit about 5-fold neutralizing antibodies against the antigen (Spike) compared to adenoviral vectors (Khoury et al., 2021). mRNA-LNP may deliver mutant p53 better than outdated delivery approaches. 4) Will enhanced immunogenicity of p53 help? Structural changes in p53, via either stabilizing mutants (Boeckler et al., 2008) or adding self-assembling modules (He et al., 2021), could maximize the host's p53 expression, presentation, and immunogenicity. 5) Shall we construct one vaccine for one p53 mutant? Each amino acid substitution in p53 may alter the p53 conformation differently (Wang and Fersht, 2015; Joerger and Fersht, 2016), so the final degradation products from one p53 mutant may differ considerably from another mutant or the WT p53 that is rarely overexpressed in cancer. The human T cell repertoire is not necessarily devoid of low- and even residual high-avidity p53-specific CTLs, yet self-tolerance certainly limits the number of high-avidity CTLs binding to MHC-restricted WT p53 peptides (Theobald et al., 1997; Kuball et al., 2002). Rational design of next-generation personalized p53 vaccines requires an in-depth understanding of mutant p53 structure and function, proteolysis, B and T cell elicitation, vaccine trafficking and retention, antigen expression and presentation, germinal center reactions, and self-tolerance. Such knowledge is essential to achieve the most effective precision vaccine candidates to be tested in clinical trials, in order to reduce cancer mortality from p53 mutation.

AUTHOR CONTRIBUTIONS

SZ contributed to drafting the manuscript; CF, ZZ, and KY contributed to concept discussion and manuscript revision; YL contributed to the design and editing of the manuscript.

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REVIEW
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The Cross Talk Between p53 and mTOR Pathways in Response to Physiological and Genotoxic Stresses

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The tumor suppressor p53 is activated upon multiple cellular stresses, including DNA damage, oncogene activation, ribosomal stress, and hypoxia, to induce cell cycle arrest, apoptosis, and senescence. Mammalian target of rapamycin (mTOR), an evolutionarily conserved serine/threonine protein kinase, serves as a central regulator of cell growth, proliferation, and survival by coordinating nutrients, energy, growth factors, and oxygen levels. p53 dysfunction and mTOR pathway hyperactivation are hallmarks of human cancer. The balance between response to stresses or commitment to cell proliferation and survival is governed by various regulatory loops between the p53 and mTOR pathways. In this review, we first briefly introduce the tumor suppressor p53 and then describe the upstream regulators and downstream effectors of the mTOR pathway. Next, we discuss the role of p53 in regulating the mTOR pathway through its transcriptional and non-transcriptional effects. We further describe the complicated role of the mTOR pathway in modulating p53 activity. Finally, we discuss the current knowledge and future perspectives on the coordinated regulation of the p53 and mTOR pathways.

Keywords: p53, mTOR, transcription, miRNA, tumorigenesis, MDM2, post-translation

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INTRODUCTION: THE TUMOR SUPPRESSOR P53

p53, a well-known tumor suppressor, acts as a "guardian of the genome" to maintain genome stability and cellular homeostasis (Vousden and Prives, 2009; Hafner et al., 2019). Upon induction of various cellular stresses, especially DNA damage, p53 is activated to induce cell cycle arrest, apoptosis, and senescence which suppress tumorigenesis by eliminating damaged and potentially precancerous cells. p53 is the most frequently mutated gene in human cancers (Hainaut and Hollstein, 2000). Approximately 50% of human tumors carry p53 mutations, and in over 80% of the tumors the p53 pathway is dysfunctional (Ozaki and Nakagawara, 2011). As a transcription factor, p53 exerts tumor-suppressive effects by directly binding to specific DNA sequences to activate or repress the transcription of target genes, such as *MDM2* (Barak et al., 1993), *p21* (el-Deiry et al., 1993), *NOXA* (Oda et al., 2000), *PUMA* (Nakano and Vousden, 2001), and *RPS27L* (He and Sun, 2007; Li et al., 2007) [for review, see (Fischer, 2017)]. The canonical p53 binding sequence RRRC (A/T) (A/T)GYYY$(N)_{0\text{-}13}$RRRC (A/T) (A/T)GYYY (R: A or G, Y: C or T, and N: any nucleotide) is normally located near the transcription start site (Hafner et al., 2019).

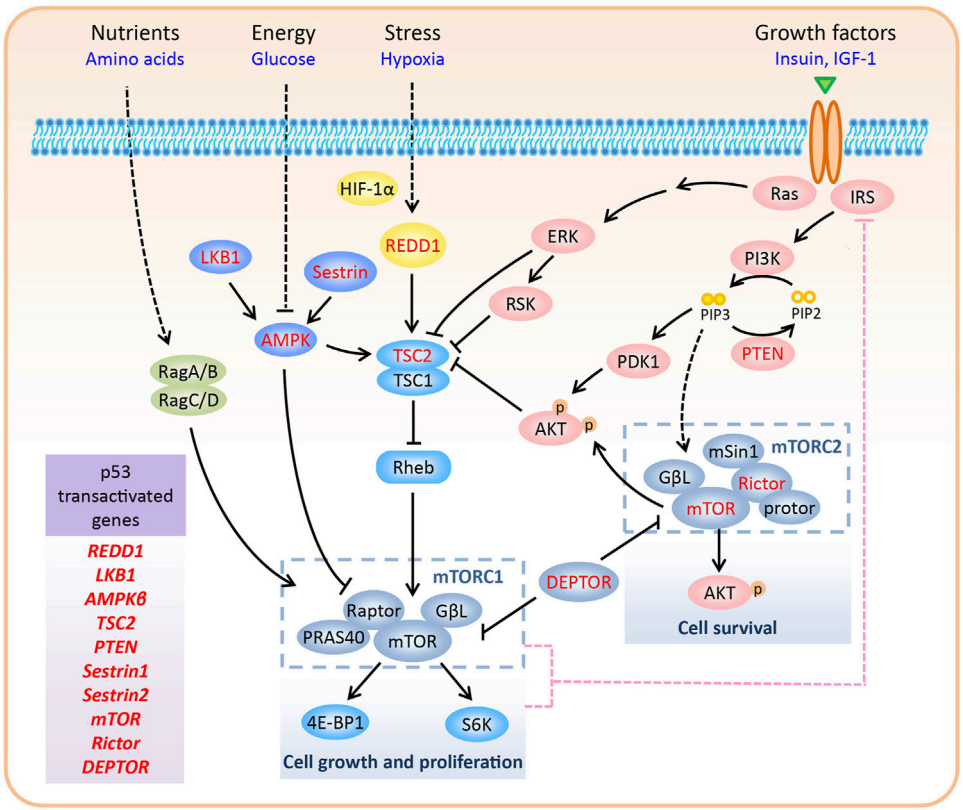

FIGURE 1 | Regulation of the mTOR pathway. In mammalian cells, mTOR forms two complexes with distinctive structure and functions, namely mTORC1 and mTORC2. mTORC1 promotes cell growth and proliferation via phosphorylation of S6K and 4E-BP1. In contrast, mTORC2 regulates cell survival by phosphorylating Ser473 of AKT. mTORC1 responds to various signals, including nutrients, energy, growth factors, and oxygen levels. Upon negative feedback from mTORC1/S6K to IRS1/PI3K signaling, hyper-activated mTORC1 inhibits the activity of mTORC2. Various components of the mTOR pathway are directly transactivated by p53, including REDD1, LKB1, AMPKβ, TSC2, PTEN, Sestrin1/2, mTOR, Rictor, and DEPTOR (highlighted in red color in the figure).

Given the key role of p53 in tumorigenesis, the protein levels and activity of p53 are precisely regulated by multiple regulators [for review, see (Kruse and Gu, 2009; Chao, 2015)], among which the E3 ubiquitin ligase, mouse double minute 2 homolog (MDM2), is the pivotal negative regulator (Hock and Vousden, 2014). Under non-stressed conditions, MDM2 binds specifically to p53 and promotes the ubiquitination and proteasomal degradation of p53, which maintains p53 at low levels. In response to stress signals, such as DNA damage, certain kinases, such as ATM and CHK2, are activated to phosphorylate p53 and abolish the interaction between p53 and MDM2, leading to p53 stabilization and transcriptional activation or repression of downstream target genes. When DNA repair is completed, p53 returns to basal levels, and subsequently, the cell cycle is restored to normal progression. The E3 ligase F-box and WD repeat domain-containing 7 (FBXW7) plays an important role in p53 turnover during DNA damage recovery (Galindo-Moreno et al., 2019; Tripathi et al., 2019; Cui et al., 2020b).

p53 dysfunction and hyperactivation of the mTOR pathway are hallmarks of human cancer (Hanahan and Weinberg, 2011). Accumulating evidence indicates that the tumor suppressor p53 regulates the machinery of the mTOR pathway at multiple levels to control a broad array of cellular processes, including cell proliferation, apoptosis, autophagy, migration, and tumorigenesis. In this review, we summarize the current knowledge regarding the role of p53 in the regulation of the mTOR pathway through transcription-dependent and transcription-independent mechanisms. A thorough understanding of the interplay between p53 and the mTOR pathway will shed light on the development of novel strategies for cancer therapy.

The mTOR Pathway

The mammalian target of rapamycin (mTOR), an evolutionarily conserved serine/threonine protein kinase, is a member of the phosphoinositide-3-kinase (PI3K)-related kinase (PIKK) family, along with ATM, ATR, DNA-PK, and SMG-1 (Lovejoy and Cortez, 2009). While all members of the family are involved in DNA damage response, mTOR also responds to various other signals, including nutrients, energy, growth factors, and oxygen levels. By integrating both extracellular and intracellular signals, mTOR coordinates cellular anabolic and catabolic processes, including cell growth, proliferation, survival, and autophagy (**Figure 1**).

mTOR and its Effectors

In mammalian cells, mTOR forms two complexes with distinctive structures and functions, namely mTORC1 and mTORC2. mTORC1 is composed of mTOR, Raptor, PRAS40, and GβL, whereas mTORC2 consists of mTOR, Rictor, mSin1, protor, and GβL (Zhao and Sun, 2012; Saxton and Sabatini, 2017). Although the mTOR protein, which is present in both the complexes, was discovered as an interacting protein of the rapamycin-FKBP12 complex, only mTORC1 is sensitive to rapamycin inhibition (Sabatini et al., 1994). Recently, the core structure of mTORC2 has been resolved, and it has been shown that the rapamycin-FKBP12 binding domain in mTOR is masked by the C-terminus of Rictor, thus resolving the rapamycin insensitivity of mTORC2 (Scaiola et al., 2020). DEP domain-containing mTOR-interacting protein (DEPTOR) inhibits mTORC1 and mTORC2 by directly binding to mTOR via its PDZ domain (Peterson et al., 2009). mTORC1 has two major substrates, S6K and 4E-BP1, which regulate several aspects of mRNA translation (Sengupta et al., 2010). Thus, mTORC1 promotes cell growth and proliferation by modulating phosphorylation-dependent mRNA translation. However, the effectors of mTORC2 are primarily AGC kinases, including AKT, PKC, and SGK1 (Fu and Hall, 2020). mTORC2 regulates cell survival by phosphorylating Ser473 of AKT, the best-characterized mTORC2 substrate. Compared to mTORC1, much is still unknown about the upstream regulators of mTORC2 (**Figure 1**). Thus, in the next section, we focus on the regulation of mTORC1 in response to various signals.

Regulation of mTORC1 by Upstream Signals

Upstream signals regulate mTORC1 activity mainly through two mechanisms: by direct control of mTORC1 components or by the supervision of Rheb GTPase, which interacts with and activates mTORC1 (Long et al., 2005). Tuberous sclerosis complex 2 (TSC2), a GTPase-activating protein (GAP) for Rheb, together with its partner TSC1, inactivates Rheb GTPase (Inoki et al., 2003). Below, we describe the manner in which mTORC1 is either activated or inactivated by distinct upstream signals.

1) Growth factors: Growth factors, such as insulin and insulin-like growth factor 1 (IGF-1), promote cellular anabolic processes by activating mTORC1. The binding of insulin/IGF-1 to its receptor recruits insulin substrate 1 (IRS1) to the receptor and activates PI3K, which phosphorylates PIP2 to PIP3. PIP3 recruits AKT to the plasma membrane, where it is fully activated by direct phosphorylation of Thr308 by PDK1 and Ser473 by mTORC2. During growth factor signaling, AKT (Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002), along with other kinases such as ERK and RSK (Roux et al., 2004; Ma et al., 2005), phosphorylates and inhibits TSC2, leading to the activation of Rheb and mTORC1. Growth factor-activated AKT also stimulates mTORC1, independent of TSC2. AKT can directly phosphorylate proline-rich AKT substrate of 40 kDa (PRAS40), a negative regulator of mTORC1, that inhibits the interaction between mTORC1 and its substrates, and dissociates itself from mTORC1 (Sancak et al., 2007; Haar et al., 2007).

2) Glucose and energy: Glucose deprivation decreases glycolytic flux and inhibits mTORC1 by lowering ATP levels. LKB1 and AMPK are two major upstream kinases of the mTOR pathway and are involved in monitoring the levels of glucose and energy (ATP and AMP) (Inoki et al., 2003; Shaw et al., 2004; Gwinn et al., 2008). In response to increased AMP/ATP ratio, LKB1 phosphorylates AMPKα (the catalytic subunit of AMPK) on Thr172 to activate AMPK. Activated AMPK phosphorylates TSC2 (Inoki et al., 2003) and Raptor (Gwinn et al., 2008) to inhibit mTORC1.

3) Hypoxia: Under hypoxic conditions, HIF-1α promotes the expression of REDD1 to inhibit mTORC1 by activating the TSC1-TSC2 complex (Brugarolas et al., 2004). Mechanistically, REDD1 may release TSC2 from its inhibitory protein 14-3-3, thus facilitating the interaction between TSC1 and TSC2 (DeYoung et al., 2008; Vega-Rubin-de-Celis et al., 2010). In addition to hypoxia, REDD1 is induced by several cellular stressors, including reactive oxygen species (ROS), glucocorticoids, DNA damage, and heat shock (Ellisen et al., 2002; Wang et al., 2003), indicating a universal function of REDD1 in coordinating stress signals to mTORC1.

4) Amino acid: Amino acid signaling recruits and activates mTORC1 to the lysosomal membrane, where a pool of Rheb resides. In this process, the Rag GTPase heterodimer (consisting of RagA/B and RagC/D), which is activated by amino acids, serves as a scaffold for mTORC1 via interacting with Raptor (Sancak et al., 2008; Sancak et al., 2010).

Feedback Loop Between mTORC1 and mTORC2

As a negative regulator of IRS1, mTORC1 also acts upstream of the PI3K-AKT pathway to inhibit mTORC2. This negative feedback loop is initiated through multiple mechanisms. First, S6K, a substrate of mTORC1, decreases the activity and protein levels of IRS1 by phosphorylating it (Harrington et al., 2004; Um et al., 2004; Shah et al., 2004). Second, mTORC1 phosphorylates and stabilizes growth factor receptor-bound protein 10 (Grb10), which inhibits IRS1/2 phosphorylation and destabilizes IRS1 (Hsu et al., 2011; Yu et al., 2011). Third, mTORC1 directly phosphorylates IRS1 at sites that inhibit its interaction with PI3K (Tzatsos, 2009). Therefore, high levels of DEPTOR, a natural inhibitor of both mTORC1 and mTORC2, inhibit mTORC1 and activate mTORC2 by relieving the feedback inhibition from mTORC1 to IRS1/PI3K signaling (Peterson et al., 2009; Zhao et al., 2011; Zhao and Sun, 2012; Cui et al., 2020a) (**Figure 1**).

Regulation of the mTOR Pathway by p53

To maintain normal cell growth and proliferation, it is important for cells to coordinate stimulatory signals (such as nutrients, energy, and growth factors) and inhibitory stresses (such as DNA damage and hypoxia). The tumor suppressor p53, a stress-induced transcription factor, can inhibit cell growth and proliferation via its target genes, such as the cyclin-dependent kinase (CDK) inhibitor p21, which serves as a cell cycle inhibitor (Levine, 1997). More recently, elucidating the mechanism of p53 in directly regulating the mTOR pathway has become an attractive area of research due to the critical roles of p53 and mTOR in tumorigenesis. In the following sections, we discuss the emerging roles of p53 in controlling the mTOR pathway through its transcriptional and non-transcriptional effects.

TABLE 1 | Target genes of p53 involved in mTOR signaling.

Targets	Position	Sequence	Refs
REDD1	−601 ~ −582	AAACAAGTCTTTCCTTGATC	Ellisen et al. (2002)
LKB1	−108 ~ −88	AACCAACGGGTGGGCACGTCG	Co et al. (2014); Xie et al. (2017)
AMPKβ	Exon 1	GTTCTTGCCGCGGCTTGCCT	Feng et al. (2007)
TSC2	Intron 2a	AGGCTAGTCTGAAACTCCTGGGCTGACGTGAC	Feng et al. (2007)
	Intron 2b	GGGCATGGTGGCACATGCCT	
	Intron 11	TAACAAGCTCGGGGCTAGCCC	
PTEN	−1190 ~ −1157	GAGCAAGCCCCAGGCAGCTACACTGGGCATGCTC	Stambolic et al. (2001)
Sestrin1 (PA26)	−1241 ~ −1222	GGACAAGTCTCCACAAGTCA	Velasco-Miguel et al. (1999)
Sestrin2 (Hi95)	Not identified	Not identified	Budanov et al. (2002)
mTOR	0.5 kb upstream promoter	Not identified	Ge et al. (2019)
Rictor	0.5 kb upstream promoter	Not identified	Ge et al. (2019)
DEPTOR	−196 ~ −169	GCTCAAGTTTCTGGGGCCGGACTAGCCC	Cui et al. (2020a)

Target Genes of p53 in the mTOR Pathway

Upon multiple stresses, p53 is activated to inhibit cell growth and proliferation, which undergo high error rates upon induction of stress. Thus, inhibition of mTORC1, which promotes cell growth and proliferation, is an important hallmark of the cellular stress response. Actually, multiple negative regulators of mTORC1, as discussed earlier, are direct transcriptional targets of p53, including *REDD1* (Ellisen et al., 2002), *LKB1* (Co et al., 2014; Xie et al., 2017), *AMPKβ* (a regulatory subunit of AMPK) (Feng et al., 2007), and *TSC2* (Feng et al., 2007). Moreover, *PTEN*, which encodes a phosphatase that catalyzes PIP3 to PIP2 to inactivate the PI3K-AKT pathway, contains p53 binding sites and is transactivated by p53 (Stambolic et al., 2001). Finally, upon genotoxic stress, p53 also promotes the transcription of Sestrin1 and Sestrin2 to inhibit mTORC1, through activation of AMPK and TSC2 (Budanov and Karin, 2008) (**Figure 1** and **Table 1**).

Contrary to the inhibitory effect of p53 target genes in controlling mTORC1, the function of these genes in mTORC2 seems much more complex, being highly cell- and context-dependent. In the alternative lengthening of telomeres (ALT) cancer cells (such as U2OS cells), p53 stimulates the transcription of mTOR and Rictor, two important components of mTORC2, to activate AKT and inhibit apoptosis (Ge et al., 2019). Recently, our group reported that DEPTOR is a direct target of p53 and its expression is positively correlated with p53 activity, both in cultured cancer cells and mouse tissues under normal conditions, and is further induced by activated p53 under genotoxic conditions. Given that DEPTOR inhibits both mTORC1 and mTORC2, and there is a negative feedback from mTORC1 to IRS1/PI3K signaling, p53-mediated DEPTOR expression has distinct roles in regulating mTORC2 under non-stressed and genotoxic stress conditions. In non-stressed cells, p53-mediated DEPTOR expression inhibits mTORC2 activity, which is reflected by the decreased phosphorylation of AKT at Ser473; whereas, upon genotoxic treatment, the dramatic induction of DEPTOR expression via p53 hyperactivation inhibits mTORC1, subsequently alleviating the feedback inhibition from mTORC1 to IRS1, thereby activating mTORC2 via IRS1/PI3K signaling (Cui et al., 2020a) (**Figure 1** and **Table 1**).

p53 Regulates the mTOR Pathway via microRNAs

The discovery of microRNAs (miRNA or miR) has added another layer of complexity to the regulation of the mTOR pathway by p53. miRNAs are a class of endogenously expressed small non-coding RNAs (17–24 nucleotides) that regulate the expression of multiple genes at the post-transcriptional level (Macfarlane and Murphy, 2010). miRNAs inhibit protein expression by enhancing mRNA degradation or suppressing translation via partial base pairing with the 3′-untranslated region (3′-UTR) of the target mRNA of protein coding genes (Lewis et al., 2005). miRNAs play critical roles in several biological processes, including proliferation, survival, metastasis, and stemness. In particular, overexpression of oncogenic miRNAs or downregulation of tumor-suppressive miRNAs contributes to tumorigenesis (Babashah and Soleimani, 2011). It is well established that p53 is an important regulator of miRNAs (Hermeking, 2007). Global sequence analysis showed that more than 46% of the 326 miRNA promoters contain putative p53 binding sites in HCT116 cells (Xi et al., 2006). In addition, various miRNAs have been identified as direct transcriptional targets of p53 and many of them are involved in p53-mediated tumor-suppressive functions (Hermeking, 2012). Moreover, besides the regulation of miRNAs at the transcriptional level, p53 promotes the maturation of certain miRNAs at the post-transcriptional level (Suzuki et al., 2009). Conversely, many miRNAs directly downregulate p53 protein levels by binding to the 3′-UTR of p53 mRNA (Liu et al., 2017).

Accumulating evidence shows that a large number of miRNAs act opposingly on the mTOR pathway, which is often hyperactivated in cancers (Zhang et al., 2017). Thus, p53 exerts its control on the mTOR pathway via miRNAs. Among all miRNAs, members of the miRNA-34 family (miR-34a/b/c) have been identified as the most common targets of p53 with the highest induction by activated p53 (Hermeking, 2007). Overexpression of miR-34a in prostate cancer cells inhibited the phosphorylation of AMPK and upregulated the phosphorylation of mTOR. As a result, miR-34a sensitizes cancer cells to chemotherapy by inhibiting autophagy through the AMPK-mTOR axis (Liao et al., 2016). However, the direct

TABLE 2 | p53 regulates the mTOR pathway via microRNAs.

miRNAs	p53-mediated regulation of miRNA	The effects of p53 on miRNA expression	miRNA targets in mTOR signaling	The effects on mTOR signaling	Refs
miR-34	Transcriptional	Upregulation	Unknown	Activation	Hermeking. (2007); Liao et al. (2016)
miR-100	Transcriptional	Downregulation	mTOR	Suppression	Sun et al. (2013); Xu et al. (2013); Zhang et al. (2014a); Ghose and Bhattacharyya (2015)
miR-101	Post-transcriptional	Upregulation	mTOR	Suppression	Lin et al. (2014); Fujiwara et al. (2018)
miR-145	Transcriptional	Upregulation	S6K1	Suppression	Sachdeva et al. (2009); Xu et al. (2012)
miR-149	Transcriptional	Upregulation	AKT1	Suppression	Jin et al. (2011); Zhang et al. (2014b)
miR-155	Transcriptional	Upregulation	Rheb, Rictor, and S6K2	Suppression	Wang et al. (2013); Wan et al. (2014); Wang et al. (2018)
miR-199a-3p	Post-transcriptional	Upregulation	mTOR	Suppression	Fornari et al. (2010); Wang et al. (2012a); Wu et al. (2013)

target(s) of miR-34 in regulating the mTOR pathway remains to be elucidated. Currently, some other miRNAs, directly regulated by p53, are emerging as vital regulators of the mTOR pathway at the post-transcriptional level, and these miRNAs, downstream of p53, are as follows (**Table 2**):

1) miR-100: miR100, a member of the miR-99 family (including miR-99a, miR-99b, and miR-100), is negatively regulated by p53. p53 binds to the upstream sequences of miR-100 and suppresses its transcription in both mouse striatal cells and human cervical carcinoma HeLa cells (Ghose and Bhattacharyya, 2015). miR-100 inhibits the expression of mTOR by directly targeting its 3′-UTR and acts as a tumor suppressor in esophageal squamous cell carcinoma (ESCC) (Sun et al., 2013; Zhang N. et al., 2014) and bladder cancer (Xu et al., 2013). Thus, p53 may activate the mTOR pathway by inhibiting the transcription of miR-100 in certain types of cancer.

2) miR-101: miR-101 is downregulated in various cancers, including ovarian cancer, prostate cancer, hepatocellular carcinoma, bladder transitional cell carcinoma, gastric cancer, and non-small cell lung cancer, and is negatively associated with the progression and invasion of malignancies, such as prostate cancer (Gui and Shen, 2012). In human osteosarcoma cells, miR-101 directly targets mTOR and decreases its expression, resulting in the suppression of cell proliferation and induction of apoptosis (Lin et al., 2014). Interestingly, p53 promotes the maturation of miR-101 at the post-transcriptional level (Fujiwara et al., 2018). Therefore, p53 may inhibit the mTOR pathway by post-transcriptional activation of miR-101.

3) miR-145: miR-145, a direct target of p53, binds to the 3′-UTR of c-MYC and inhibits its expression, thereby repressing cancer cell growth both *in vitro* and *in vivo* (Sachdeva et al., 2009). Moreover, miR-145 suppresses S6K1 expression at the post-transcriptional level to inhibit tumorigenesis and tumor angiogenesis (Xu et al., 2012). Furthermore, p53-mediated transcription of miR-145 may suppress tumor growth by cooperatively inhibiting the oncogenic functions of c-MYC and the mTOR pathway.

4) miR-149: miR-149 plays a dual role, that is controversial, either as a tumor suppressor or as an oncogene in different types of cancer (Wang Y. et al., 2012). miR-149 inhibits the tumorigenesis of hepatocellular carcinoma (HCC) via directly targeting AKT1 to regulate the AKT/mTOR pathway (Zhang Y. et al., 2014). However, miR-149, which is directly upregulated by p53, acts as an oncogenic regulator in melanoma cells by targeting glycogen synthase kinase 3α (GSK3α) to stabilize MCL-1 and inhibit apoptosis (Jin et al., 2011). Thus, it will be intriguing to characterize the unique role of the p53-miR-149-AKT/mTOR axis in different types of tumors.

5) miR-155: miR-155 targets several components of the mTOR pathway, including Rheb, Rictor, and S6K2, by directly binding to their 3′-UTRs (Wang et al., 2013; Wan et al., 2014). By interfering with both mTORC1 and mTORC2 signals, miR-155 suppresses cell proliferation, activates autophagy, and induces G1/S cell cycle arrest. Additionally, it has been reported that under high glucose conditions, p53 directly promotes miR-155 expression as a transcription factor in human renal proximal tubule (HK-2) cells (Wang et al., 2018). However, the role of p53 in regulating the transcription of miR-155 under normal conditions or upon glucose deprivation, and the functions of the p53-miR-155-mTOR pathway in physiological and pathological processes remain largely unknown.

6) miR-199a-3p: miR-199a-3p is upregulated by p53 at the post-transcriptional level (Wang J. et al., 2012). miR-199a-3p directly interacts with the 3′-UTR of mTOR and inhibits the mTOR pathway and restrains endometrial cancer cell proliferation (Wu et al., 2013) as well as increases the sensitivity of HCC cells to doxorubicin-induced apoptosis (Fornari et al., 2010). Given that p53 is highly activated in response to DNA damage, due to genotoxic treatments (e.g., doxorubicin), it is probable that the p53-miR-199a-3p-mTOR pathway regulates cancer cell survival during chemotherapy or radiotherapy.

Non-Transcriptional Effects of p53 in Regulating the mTOR Pathway

In general, the tumor suppressor p53 regulates various cellular processes via *trans*-activating or *trans*-repressing downstream gene expression as a transcription factor. Interestingly, in recent decades, several studies have shown that in addition to its activity in the nucleus, p53 exhibits transcription independent functions in the cytoplasm (Comel et al., 2014). The best-characterized

extranuclear function of p53 is the induction of apoptosis. It has been reported that overexpression of a truncated murine p53 (p53dl214), containing only 214 amino acid residues of the N-terminus and lacking DNA-binding activity, could trigger extensive apoptosis in HeLa cells as well (Haupt et al., 1995). Mechanistically, upon apoptotic induction, p53 translocates from the nucleus to the mitochondrial outer membrane, and interacts with pro-survival Bcl-2 family members (such as Bcl-w and Bcl-X_L) to release pro-apoptotic Bcl-2 proteins (such as Bax and Bak) to induce apoptosis (Vaseva and Moll, 2009; Czabotar et al., 2014). Moreover, cytosolic p53 regulates autophagy via the mTOR pathway.

Autophagy, a cellular catabolic process that recycles unwanted proteins and damaged organelles in the lysosomes, is regulated by two biologically significant molecules: mTOR and AMPK. mTORC1 inhibits autophagosome formation by phosphorylating ULK1 at Ser757 to suppress the ULK1 complex (Kim et al., 2011), and mTORC2 restrains the transcription of several *ATG*s via AKT-FoxO3 signaling to inhibit autophagy (Guertin et al., 2006; Zhao et al., 2008). However, AMPK plays a positive role in autophagy induction. On one hand, AMPK can phosphorylate TSC2 and Raptor to inhibit mTOR (Inoki et al., 2003; Gwinn et al., 2008); on the other hand, AMPK can directly phosphorylate ULK1 at Ser317 and Ser777 to activate the ULK1 complex and initiate autophagy (Kim et al., 2011). Notably, the tumor suppressor p53 has a dual role in the regulation of autophagy. As a transcription factor, p53 transactivates several genes that induce autophagy, including *TSC2* (Feng et al., 2007), *AMPKβ1* (Feng et al., 2007), *Sestrin1/2* (Budanov and Karin, 2008), and *DRAM* (Crighton et al., 2006). However, cytosolic p53, either the wild-type or mutant form, represses autophagy (Tasdemir et al., 2008a; Tasdemir et al., 2008b). In fact, various known autophagy-inducing stimuli, such as rapamycin treatment or ER stress, cause the cytoplasmic translocation of p53, which is subsequently degraded via MDM2-mediated ubiquitination (Pluquet et al., 2005; Yorimitsu et al., 2006). Consistently, pharmacological inhibition or depletion of p53 induces autophagy in nematodes, mice, and human cells under normal conditions (Tasdemir et al., 2008b). Moreover, p53 suppresses autophagy through a non-transcriptional effect via cytoplasmic localization, which is supported by the following evidence: 1) expression of both wild-type and ER-targeted p53 inhibited high levels of basal autophagy in HCT116 $p53^{-/-}$ cells; 2) expression of nuclear p53 (disturbed NES by L348A and L350A) failed to inhibit autophagy; and 3) a point mutation (R175H) in p53 that induces a conformational change, abrogated the autophagy-inhibitory effect of p53 (Tasdemir et al., 2008b). However, the exact molecular mechanism by which cytoplasmic p53 inhibits autophagy remains to be elucidated. It seems that cytoplasmic p53 inhibits autophagy through a mechanism different from its function in apoptosis, since BH3-only proteins (such as Beclin-1) are autophagy inducers, but not suppressors (Maiuri et al., 2007). Current research highlights the involvement of the mTOR pathway in regulating cytoplasmic p53-induced autophagy. In HCT116 $p53^{-/-}$ cells with higher basal levels of autophagy, S6K, an mTOR substrate, was hypophosphorylated, whereas AMPK and its substrate ACC were hyperphosphorylated. In addition, cytoplasmic, but not nuclear, p53 inhibited AMPK (reflected by reduced phosphorylation of AMPK, ACC, and TSC2) and activated mTOR (reflected by an increased phosphorylation of S6K) to suppress autophagy (Tasdemir et al., 2008b). These results indicate that cytoplasmic p53 can regulate the AMPK-mTOR axis, but the molecular details remain unclear. Since cytoplasmic p53 can regulate cellular processes by modulating protein-protein interactions, it is important to identify novel cytoplasmic p53-binding proteins, which are involved in controlling mTOR activity, to uncover the exact role of cytoplasmic p53 in regulating the mTOR pathway.

Regulation of p53 by the mTOR Pathway

The coordination of growth signals and stresses is also adjusted by the reverse regulation of p53 by the mTOR pathway. The role of the mTOR pathway in regulating p53 activity is complex, and is mainly focused on modulating the protein levels of p53 and/or MDM2, a negative regulator of p53. Activating the mTOR signaling by growth factors, such as IGF-1 and hepatocyte growth factor (HGF), induces MDM2 translation in a PI3K-AKT dependent manner. On the other hand, inhibition of mTORC1 by rapamycin downregulating MDM2, induces p53-dependent apoptosis, and sensitizes cancer cells to chemotherapy (Moumen et al., 2007; Du et al., 2013). Additionally, the treatment of Torin1 (an inhibitor of both mTORC1 and mTORC2) or PF-04691502 (a dual PI3K/mTOR inhibitor) increases the expression of p53 protein via inhibition of mTOR signaling (Ekshyyan et al., 2013; Herzog et al., 2013; Garbern et al., 2020). However, in Tsc1 or Tsc2 deletion MEF cells, constitutive mTOR activation promotes the association of p53 mRNA with polysomes to induce its translation (Lee et al., 2007).

In addition to the modulation of p53 activity via changing MDM2 or p53 protein synthesis, the mTOR signaling regulates p53 activity at post-translational levels. First, in PTEN-depleted cells, mTORC1 and mTORC2 compete with MDM2 to bind p53 and phosphorylate it at Ser15, which is the first step to activate p53, leading to PTEN-loss-induced cellular senescence (PICS) (Jung et al., 2019). However, PTEN activates p53 and sensitizes tumor cells to chemotherapy by retaining MDM2 in the cytoplasm (Mayo et al., 2002). And PTEN also promotes p53 transcription activity by regulating its DNA binding independent of MDM2 (Freeman et al., 2003). Second, upon glucose deprivation, phosphorylation of p53 at Ser15 by AMPK leads to cell cycle arrest (Jones et al., 2005). Furthermore, LKB1, the upstream activator of AMPK, directly or indirectly phosphorylates p53 at Ser15 and Ser392, and activates the transcription of *p21* following UV treatment (Zeng and Berger, 2006). Third, AKT promotes p53 degradation by directly phosphorylating MDM2 on Ser166 and/or Ser186, which facilitates the nuclear translocation of MDM2 (Mayo and Donner, 2001; Zhou et al., 2001) and stabilizes it (Feng et al., 2004). Particularly, *in vivo* studies using $Mdm2^{S183A}$ mice recently showed that AKT phosphorylation of Mdm2 at Ser183 (the murine equivalent of human Ser186) suppresses p53-mediated senescence, facilitates ROS-induced tumorigenesis,

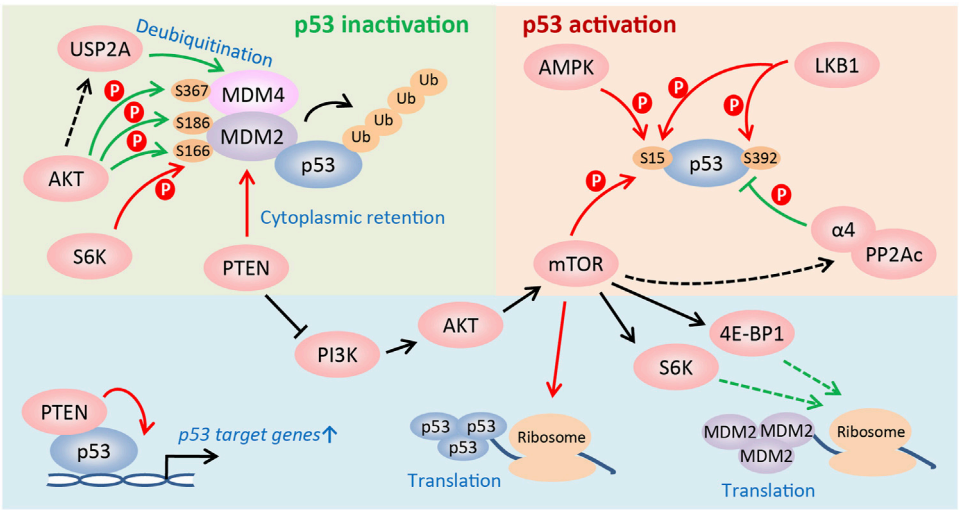

FIGURE 2 | Regulation of p53 activity by the mTOR pathway. The PI3K/AKT/mTOR pathway regulates the activity of p53 at multiple levels, such as translational and post-translational levels, etc. The mTOR pathway is able to either positively or negatively regulate p53 activity in a cell type and stress-dependent manner. See text for details (Red arrow: to promote p53 activity; green arrow: to suppress p53 activity).

and has no effects on DNA damage response induced by radiation (Chibaya et al., 2021). Moreover, AKT also regulates phosphorylation of MDM4, which complexes with MDM2 to degrade p53, at Ser367 to stabilize it and consequently inactivate p53 (Lopez-Pajares et al., 2008; Pellegrino et al., 2014). Additionally, AKT may protect MDM4 from proteolysis by inducing the ubiquitin-specific protease 2a (USP2A) to deubiquitinate it (Allende-Vega et al., 2010; Calvisi et al., 2011; Pellegrino et al., 2014). Fourth, in addition to phosphorylating MDM2 on Ser166, S6K1 interacts strongly with MDM2 and inhibits MDM2-mediated p53 degradation in response to DNA damage (Lai et al., 2010). Finally, α4, a nonanalytic subunit of protein phosphatase 2A (PP2A), dephosphorylates p53 and suppresses apoptosis by inhibiting expression of p53 target genes, such as NOXA and p21 (Kong et al., 2004). Rapamycin treatment disrupts the association of α4 with PP2Ac, the catalytic subunit of PP2A, to suppress the phosphatase activity of PP2A (Murata et al., 1997; Inui et al., 1998; Kong et al., 2004), indicating that activating mTOR signaling promotes p53 dephosphorylation and represses its activity. Collectively, the mTOR pathway is able to either positively or negatively regulate p53 activity in a cell type- and stress-dependent manner (**Figure 2**).

CONCLUSIONS AND FUTURE PERSPECTIVES

In summary, the coordinated regulation of the tumor suppressor p53 and the mTOR pathway is critical for cells and organisms to maintain homeostasis in response to various stimuli. p53 controls the mTOR pathway at multiple levels: 1) p53 directly regulates several signaling mechanisms in the mTOR pathway; 2) miRNAs, downstream of p53, regulate the mTOR pathway at the post-transcriptional level; 3) cytoplasmic p53 may control the AMPK-mTOR axis to inhibit autophagy by protein-protein interaction. In contrast, the mTOR pathway regulates p53 activity mainly by monitoring the interaction between p53 and its E3 ubiquitin ligase, MDM2. Although some cross talk between p53 and the mTOR pathway has been addressed, many fundamental questions remain unanswered such as: 1) Are the other regulators or components of the mTORC1 complex, the transcriptional targets of p53? 2) How does cytoplasmic p53 activate AMPK and suppress mTOR? 3) What is the precise role of p53 in regulating mTORC2, which has been poorly studied? 4) Does mTOR or its downstream effectors directly phosphorylate other sites of p53 in addition to Ser15 and regulate p53 function under physiological or "stress" conditions? 5) Do the *in vitro* findings truly indicate those *in vivo* physiological and pathological conditions (genetically modified mouse models and clinical patient samples)? The answers to these questions will advance our current understanding of the manner in which the cross talk between p53 and mTOR pathways regulates tumorigenesis.

Interestingly, activating p53 and inhibiting mTOR may be an effective strategy for combating coronaviruses (CoVs) such as COVID-19-causing SARS-CoV-2. During viral infection and replication, mTOR is activated and promotes type-I interferon expression in the presence of MyD88, TLR9, and IRF-7. mTOR inhibitors suppress viral infection and replication in the early stages (Ramaiah, 2020). Recent studies have indicated that several mTOR inhibitors, such as rapamycin and metformin, are potential COVID-19 inhibitors (Gordon et al., 2020; Sharma et al., 2020). However, p53 is an anti-viral factor that is degraded by the E3 ubiquitin ligase RCHY1 upon SARS-CoV infection (Ma-Lauer et al., 2016). Since p53 target genes in the mTOR pathway are mainly negative regulators of mTORC1, p53 activators, such as Nutlin-3a, could help in inhibiting SARS-CoV-2 replication by suppressing mTOR activity.

AUTHOR CONTRIBUTIONS

DC drafted the manuscript. RQ, DL, XX and TL revised the manuscript. YZ conceptualized, revised, and finalized the manuscript.

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Oligomerization of Mutant p53 R273H is not Required for Gain-of-Function Chromatin Associated Activities

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The *TP53* gene is often mutated in cancer, with missense mutations found in the central DNA binding domain, and less often in the C-terminal oligomerization domain (OD). These types of mutations are found in patients with the rare inherited cancer predisposition disorder called Li-Fraumeni syndrome. We previously found that mutant p53 (mtp53) R273H associates with replicating DNA and promotes the chromatin association of replication-associated proteins mini-chromosome maintenance 2 (MCM2), and poly ADP-ribose polymerase 1(PARP1). Herein, we created dual mutants in order to test if the oligomerization state of mtp53 R273H played a role in chromatin binding oncogenic gain-of-function (GOF) activities. We used site-directed mutagenesis to introduce point mutations in the OD in wild-type p53 (wtp53), and mtp53 R273H expressing plasmids. The glutaraldehyde crosslinking assay revealed that both wtp53 and mtp53 R273H formed predominantly tetramers, while the single OD mutant A347D, and the dual mtp53 R273H-A347D, formed predominantly dimers. The R337C, L344P, mtp53 R273H-R337C, and mtp53 R273H-L344P proteins formed predominantly monomers. Wtp53 was able to activate the cyclin-dependent kinase gene *p21/waf* and the p53 feedback regulator *MDM2*. As expected, the transactivation activity was lost for all the single mutants, as well as the mtp53 R273H-dual mutants. Importantly, mtp53 R273H and the dual oligomerization mutants, R273H-A347D, R273H-R337C, and R273H-L344P were able to interact with chromatin. Additionally, the dual oligomerization mutants, R273H-A347D, R273H-R337C, and R273H-L344P, maintained strong interactions with MCM2 and PARP1. Our findings suggest that while mtp53 R273H can form tetramers, tetramer formation is not required for the GOF associated chromatin interactions.

Keywords: mutant p53, oligomerization domain, gain-of-function, chromatin, replication-associated

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INTRODUCTION

Cancers often have genetic mutations in the *TP53* gene that can be both inherited and spontaneous (Levine, 2021). These mutations often disrupt the sequence-specific DNA binding activity of wild-type p53 (wtp53) and can also be found, albeit less frequently, in the C-terminal oligomerization domain (OD) (Levine, 2021). A subset of *TP53* mutations transform the gene into an oncogene, producing a class of mutant p53 (mtp53) proteins known to have gain-of-function (GOF) properties

that help in tumor promotion (Weisz et al., 2007; Sullivan et al., 2018). The GOF mtp53 proteins possess biochemical properties distinct from wtp53, including longer half-lives, transcriptional activation of non-canonical p53 target genes, and exhibit stable complex formation with both canonical and non-canonical protein interaction partners (Sabapathy, 2015; Sabapathy and Lane, 2018). Amino acids in the central region of wild-type p53 form direct DNA contacts and conformationally coordinate the protein for stable sequence-specific interactions at p53 response elements; the hotspot cancer amino acid mutations in Arg 248 (R248) and Arg 273 (R273) correspond to amino acid residues that make direct contact with the DNA backbone (Cho et al., 1994). The fact that p53 functions as a tetramer was discovered in part by glutaraldehyde cross-linking experiments that examined the entire protein (Friedman et al., 1993). Crystal structure studies exclusively with the OD further clarified how critical p53 amino acids affect tetramer formation (Friedman et al., 1993; Jeffrey et al., 1995).

The OD of p53 is composed of amino acid residues stretching from position 323-355 that assist in sequence specific DNA binding and transcriptional activation (Waterman et al., 1995). The OD of p53 is comprised of a ß-strand (Glu326-Arg333), a tight turn (Gly334), and an α-helix (Arg335-Gly355) (Jeffrey et al., 1995; Kamada et al., 2016). Two monomeric p53s form a dimer when the ß-strands of their ODs interact in an anti-parallel manner. This anti-parallel ß-strand-interaction allows each dimer to have its α-helix projecting outward, allowing it to form a dimer-of-dimers with other α-helices to form a four-helix tetramer (Johnson et al., 1995). The four-helix tetramer configuration positions the DNA-binding domain close to the p53 response element for easy interaction and is required for transactivation of target genes (Weisz et al., 2007; Goh et al., 2011).

Tetramerization of wtp53 is important for tumor-suppressor activities that lead to the activation of apoptosis (Fischer et al., 2016). Compromising the ability of wtp53 to form a tetramer downregulates the expression of p53 target genes (Kawaguchi et al., 2005). In Li-Fraumeni syndrome (a condition where patients present with germline p53 mutations and have a predisposition to early-life cancer development), point mutations within the OD (R337C, A347D, or L344P) destabilize tetramer formation and decrease the ability of p53 to bind DNA, as well as activate transcription of *p21*, *Bax,* and *PUMA* (Jeffrey et al., 1995; Davison et al., 1998; Lomax et al., 1998). Interestingly, in cancers the p53 associated mutations have never been reported to occur simultaneously in the OD and in the DNA binding domain. This suggests that the OD and DNA binding domain mutations make independent contributions to p53 transactivation activity. Therefore there is no selection for dual mutants in cancers. Simply inhibiting the transcription factor function of p53, in one way or the other, would be enough to promote tumorigenesis. However, p53 is known to have functions that are separable from its transcription factor activity. For example, p53 participates in the regulation of DNA replication (Bargonetti and Prives, 2019). It may be some of these functions that are co-opted by different GOF mtp53 proteins. Recently, a C-terminal frame shift p53 mutant has been shown to gain some new functions (Tong et al., 2021). We found that mtp53 R273H associates with replicating chromatin, but it was not clear if the oligomerization of mtp53 played a role in chromatin-association (Xiao et al., 2020). With this in mind, we decided to create dual mutants (even though these do not exist in cancers). This was done in order to investigate whether the oligomerization state of mtp53 R273H influenced the DNA binding of mtp53 R273H, and the GOF associated replication activities.

GOF mtp53 R273H interacts with replicating DNA, and the replication associated proteins poly ADP-ribose polymerase 1 (PARP1), and the DNA helicase mini-chromosome maintenance complex (MCM2-7) in a mtp53-PARP-MCM axis (Polotskaia et al., 2015; Qiu et al., 2017; Bargonetti and Prives, 2019; Xiao et al., 2020). The mtp53-PARP-MCM axis on chromatin suggests a role for GOF mtp53 in DNA repair and/or replication mechanisms. Exogenous expression of GOF mtp53 (R175H or R273H) in human cells correlates with the increased transcription of DNA replicating factor *CDC7* (which increases DNA replication origin firing) (Datta et al., 2017). The transcriptional activation of previously silent genes in the presence of mtp53 in cancer cells has recently been associated with p53 mutations driving aneuploidy, rather than as a direct transcriptional response (Redman-Rivera et al., 2021).

Li-Fraumeni syndrome (LFS) predisposes patients to early onset of different types of cancer (Srivastava et al., 1990; Malkin, 1993; Malkin, 2011). In a cohort in Brazil, it was reported that the R337C mutation in the OD of p53 predisposes LFS patients to adenocarcinoma (Fischer et al., 2018). While it has already been reported that mutations in the OD region compromise wtp53 transcriptional activity, we wanted to determine if LFS associated OD mutations changed mtp53 R273H chromatin-associated activities. We used site-directed mutagenesis to introduce point mutations in plasmids expressing either wtp53 or mtp53 R273H to alter amino acids R337C, A347D, and L344P. We observed that exogenously expressed mtp53 R273H formed tetramers, and that dual mtp53 R273H-A347D formed predominantly dimers, while dual mtp53 R273H-R337C and R273H-L344P formed predominantly monomers. The destabilizing oligomerization mutations to create dual-mtp53 did not inhibit mtp53 R273H interactions with chromatin. Moreover, the interaction between mtp53 R273H and MCM2 or PARP1 were maintained in the dual-R273H oligomerization mutants. These findings suggest that oligomerization of GOF mtp53 R273H does not significantly influence GOF chromatin associated activities.

MATERIALS AND METHODS

Materials

Solvents and chemicals including DMSO, glutaraldehyde, temozolomide, and talazoparib were obtained from Sigma-Aldrich (St. Louis, MO, United States). DO1 p53 (Cat# sc-126) monoclonal and PARP1 (Cat# sc-7150) rabbit polyclonal antibodies were purchased from Santa Cruz (United States). MCM2 (Cat# 12079s) mouse antibody was purchased from

Cell Signaling Technology. An Eppendorf 5,415 refrigerated centrifuge was used for preparation of all extracts.

Cell Culture and Drug Treatments

Human breast cancer cell line MDA-MB-468 was purchased from ATCC (www.atcc.org) and the HCT116 colon cancer cell line that is p53−/− was a gift from Bert Vogelstein and were made as described (Bunz et al., 1998; Bunz et al., 1999). Cell lines were regularly authenticated via short tandem repeat technology (Genetica DNA Laboratories). Cells were routinely checked for mycoplasma contamination by PCR assay (ATCC). Fresh cells were thawed when the passaging period was around 30. Cells were maintained at 5% CO_2 in a 37°C humidified incubator. HCT116 p53−/− and MDA-MB-468 cells were cultured in McCoy's 5A (Gibco) and DMEM media (Corning) respectively, with 50 U/ml penicillin, 50 μg/ml streptomycin (Mediatech), 5 ug/ml plasmocin (InvivoGen) and supplemented with 10% FBS (Gemini). PARP-dependent recruitment of proteins to chromatin was assayed by exposure of HCT116 cells to 1 mM Temozolomide (Sigma-Aldrich; 100 mM stock solution in DMSO) and 10 μM Talazoparib (Selleckchem; 20 mM stock solution in DMSO) combination treatment for 4 h at 37°C followed by chromatin isolation as described (Qiu et al., 2017).

Site-Directed Mutagenesis, Clone Validation, and Transfection

To generate clones expressing p53 with the desired mutations in the oligomerization domain, we used the NEBasechanger (https://nebasechanger.neb.com) platform to design primers to introduce specific point mutations within the OD of pCMV-FLAG-wtp53 and pCMV-FLAG-p53R273H plasmids which express wtp53 or R273H mtp53 (Hamard et al., 2012). For R337C, the primer pair was F: TGGGCGTGAGtGCTTCGA GAT and R: CGGATCTGAAGGGTGAAATATTCTC (annealing temperature 66°C). For A347D, the primer pair was F: CTGAATGAGGaCTTGGAACTC and R: CTCTCGGAACAT CTCGAAG (annealing temperature 62°C). Lastly, for L344P the primer pair was F: TTCCGAGAGCcGAATGAGGCC and R: CATCTCGAAGCGCTCACG (annealing temperature 65°C). A PCR reaction was set up with CMV-wtp53 or CMV-R273H plasmid template and the Q5 Hotstart high-fidelity 2X master mix (NEB) at the specific annealing temperature of each primer pair. After confirming PCR amplicon with agarose gel electrophoresis, a kinase-ligase-Dpn1 (NEB) reaction was done before transforming DH5α competent cells. DNA was isolated from cultured transformants (grown in LB+50 μg/ml Amp) using the Qiagen miniprep/midiprep kit, sequenced (Genewiz) using a p53 Exon 8 F primer 5′ ACAGCACATGACGGAGGTTGT, and plasmids harboring mutations were confirmed using the Benchling™ platform. Sequences were compared to TP53 cDNA from the GRCh38 homo sapien reference genome using the benchling platform's external databases. Plasmids with the desired OD mutations were transfected into HCT116 p53−/− cells using either the lipofectamine™ (Invitrogen) or the electroporation-based Neon Transfection System™ (ThermoFisher) as directed by the manufacturers. For the nucleofection, the transfection protocol as previously described for MDA-MB-468 cells was followed with little modification (Ellison et al., 2021). The conditions for introduction of plasmids into HCT116 p53−/− cells (7 μg/1,000,000 cells) using the Neon were pulsation 1x for 20 ms at 1530 V followed by culturing in McCoy's 5A media +10% FBS without antibiotics.

Glutaraldehyde Chemical Cross-Linking Assay

This assay was carried out as described previously for MDA-MB-468 cells and purified R273H mtp53 (Ellison et al., 2021; Xiao et al., 2021). Cells were washed with cold PBS, harvested by scraping in cold PBS, and centrifuging at 1,400 *g* (1,100 rpm) for 7 min. Harvested cells were lysed with phosphate lysis buffer (1x PBS, 10% glycerol, 10 mMEDTA, 0.5% NP-40, 0.1M KCl, 1 mM PMSF, 8.5 μg/ml aprotinin, 2 μg/ml leupeptin, and phosphatase inhibitor cocktail). A total of 50 μg of protein lysates were treated without, or with, glutaraldehyde at a final concentration of 0.005%. The lysates were then incubated at room temperature for 20 min on a shaker. The crosslinking was stopped by the addition of 1/6 of the volume with 6X protein sample buffer (6X SDS Laemmli sample buffer, 0.2 M DTT) and heated at 95°C for 10 min 25 μg was resolved on an 8% SDS-PAGE gel and Western blot analysis was performed with DO1 p53 monoclonal antibody.

TaqMan Real-Time PCR

Total RNA was isolated from cell cultures as dictated by the experimental design using the Qiagen RNeasy™ kit as directed by the manufacturers (www.qiagen.com/HB-0435). The Applied Biosystems High-Capacity cDNA Reverse Transcription kit was used to generate cDNA from 5 μg of total RNA from each cell sample. The relative abundance of the *TP53, p21, MDM2, RRM2* and *CDC7* mRNA within each cell was measured by quantitative-PCR using the Applied Biosystems TaqMan real-time PCR with FAM dye-labeled probes and *GAPDH* as an endogenous control (Thermofisher Scientific *TP53* ID# 01034249_m1, *p21* cat# 4331182, *MDM2* cat# 4351372 *RRM2* cat# 01072069_g1, *CDC7* ID# 00177487_m1, and *GAPDH* ID# 02786624_g1) and the Applied Biosystems QuantStudio seven Flex instrument.

Whole-Cell Lysis and Immunoblotting Assay

Cells were harvested by low speed centrifugation at 1,400 *g* (1,100 rpm) for 7 min at 4°C. Cells were washed three times with ice-cold PBS and resuspended in RIPA buffer (0.1% SDS, 1% IGEPAL NP-40, 0.5% Deoxycholate, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM Tris-Cl pH 8.0, 1 mM PMSF, 8.5 μg/ml Aprotinin, 2 μg/ml Leupeptin and phosphatase inhibitor cocktail). The cell suspension was incubated on ice for 30 min to lyse the cells, with gentle vortexing every 5 min, after which, lysates were subject to sonication 3x for 30 s pulses/30 s rest on ice at 98% amplitude and then centrifuged at 15,700 *g* (13,200 rpm) for 30 min at 4°C. The protein concentrations of clarified cell extracts were

determined using the Bradford assay (Bio-Rad), and 50 μg of extracts were analyzed for specific proteins by electro-transfer onto Polyvinylidene fluoride membrane (Amersham-GE Biosciences) following SDS-PAGE. The membrane was blocked with 5% non-fat milk (Bio-Rad) in either 1X PBS-0.1% Tween-20 or 1X TBS-0.1% Tween-20 followed by an overnight incubation with primary antibody at 4°C. The membrane was washed 3x with either 1X PBS-0.1% Tween-20 or 1X TBS-0.1% Tween-20 and incubated with Cy5-and Cy3-linked secondary antibodies (Amersham Biosciences) for 1h at room temperature. The signal was detected with the Typhoon FLA 7000 laser scanner (GE Healthcare). Primary antibodies used were; 1) anti-p53 DO1 p53 (Santa Cruz Cat# sc-126), 2) anti-PARP1 (Santa Cruz Cat# sc-7150), 3) anti-MCM2 1E7 (Cell Signalling Technology Cat# 12079s).

Chromatin Fractionation Assay

Localization of mtp53 proteins to chromosomes was assessed using a version of the Chromatin Fractionation Assay as described (Ellison et al., 2021). Cells were harvested 24 h post-transfection as dictated by experimental conditions by scraping, pelleted, and washed with ice-cold PBS three times. The pellet was resuspended in Buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 300 mM Sucrose, 1 mM DTT, 10% Glycerol, 0.1 mM PMSF, 1 μg/ml Leupeptin, 1 μg/ml Pepstatin A, and 2 μg/ml Aprotinin) with 0.1% Triton X-100 using 3X the pellet volume. The resuspended pellet was centrifuged at 1,500 *g* (4,000 rpm) for 5 min after incubating on ice for 5 min. The resulting pellet containing nuclei was saved and the supernatant spun at 15,700 *g* (13,200 rpm) for 5 min; the supernatant from this centrifugation step was saved as S1. The nuclei from each sample were washed twice in Buffer A+ 0.15% Triton X-100 then lysed in Buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 1 μg/ml Leupeptin, 1 μg/ml Pepstatin A, and 2 μg/ml Aprotinin) on ice for 30 min. The chromatin for each sample was separated from the nuclear lysate (S2) by centrifugation for 4 min at 1,500 *g* (4,000 rpm) at 4°C, washed with Buffer B, and then collected by centrifugation as described above. The chromatin pellet was resuspended in Buffer B and sonicated on ice to shear genomic DNA, the resulting solution was saved as the chromatin fraction. The protein concentrations were measured using the Bradford assay (Bio-Rad). ImageJ quantification is shown in **Supplementary Figure S3A**. The ImageJ software was used to determine the western blot band intensity with signals normalized to their respective Lamin control. The relative expression of p53 in the HCT116−/− transfected cells was determined by using the level of R273H protein in the no drug treatment as the reference sample. The relative expression of MCM2 and PARP1, was determine by using the protein levels in the empty vector transfection sample with no drug treatment as the reference samples.

Detergent Solubility Assay

The method was derived from a DNA repair tight tethering detergent assay (Iwabuchi et al., 2003). Transfected HCT116 p53−/− cells were harvested and then stored at −80°C. For preparation of the soluble and insoluble fractions, the frozen cell pellets were lysed for 1 h at 4°C in TNE (pH 8.0) buffer (50 mM Tris, pH8.0,120 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 100 g/ml phenylmethylsulphonyl fluoride, 5 g/ml aprotinin, 5 g/ml Pepstatin, 2 g/ml leupeptin, 50 mM NaF, 2 mM $Na_3VO_4{\cdot}2H_2O$ (Sodium Orthovanadate)). Approximately 3x the cell pellet volume of TNE buffer was used for lysis. The lysate was divided into the soluble and insoluble fractions by centrifugation at 15,700 *g* (13,200 rpm) for 10 min at 4°C. The supernatant-soluble fraction was transferred to a new tube. The remaining pellet, or insoluble fraction was resuspended in PBS containing 2% SDS (~5x insoluble cell pellet volume) and then sonicated 3 times for 30 s pulses/30 s rest on ice at 98% amplitude (QSonica, LLC Q700). The protein concentration for the soluble fraction was measured using the Bradford assay, while the protein concentration of the insoluble fraction was obtained by absorbance at 280 nm reading from Nanodrop Spectrophotometer. Samples were analyzed by western blotting as described above. ImageJ quantification is shown in **Supplementary Figure S3B** and determined as described above for chromatin fractionation samples.

Immunofluorescent Assay Coupled Proximity Ligation Assay

The protocol for the proximity ligation assay using the Sigma Aldrich Duolink Kit™ (Cat # DUO92008) was as described previously (Xiao et al., 2020). Cells were seeded in a 12-well glass-bottomed plate at 1×10^5 cells per well in complete McCoy 5A media supplemented with 10% FBS without antibiotics. After 24 h, 1.6 ug of plasmid DNA was added to each well via the lipofectamine transfection system as described by the manufacturer. After 24 h post-transfection, the media was removed, and the cells were washed with cold PBS 3 times. The cells were then fixed in 4% formaldehyde for 15 min and permeabilized in 0.5% Triton X-100 for 10 min at room temperature. The Duolink *In-situ* Red Kit (Cat # DUO92101) was used for the PLA. Blocking buffer was added to each well and incubated in a humidified chamber at 37°C for 30 min. After aspirating the blocking buffer, primary antibodies were added to each well and incubated in a humidified chamber overnight at room temperature. Wash buffer A (cat # DUO82049) was used to wash the cells 3 times for 5 min each. The PLA Plus/Minus secondary antibody probes were added to the wells and incubated in a humidified chamber for 60 min at 37°C. After that, the cells were washed 2 times with buffer A for 2 min each. The ligation step was performed for 30 min incubation at 37°C, washed 2 times with buffer A and the amplification was done for 100 min at 37°C. The wells were washed with buffer B for 10 min and incubated with a FITC-labelled secondary antibody to detect p53. After washing 3 times, mounting media containing DAPI was added to each well, rocked for 15 min, and images were taken using the Nikon A1 confocal microscope. Images obtained were processed with the Nikon NIS Element software, ImageJ and Cellprofiler. The foci/cell were plotted, and statistical analysis was performed in GraphPad Prism 9.

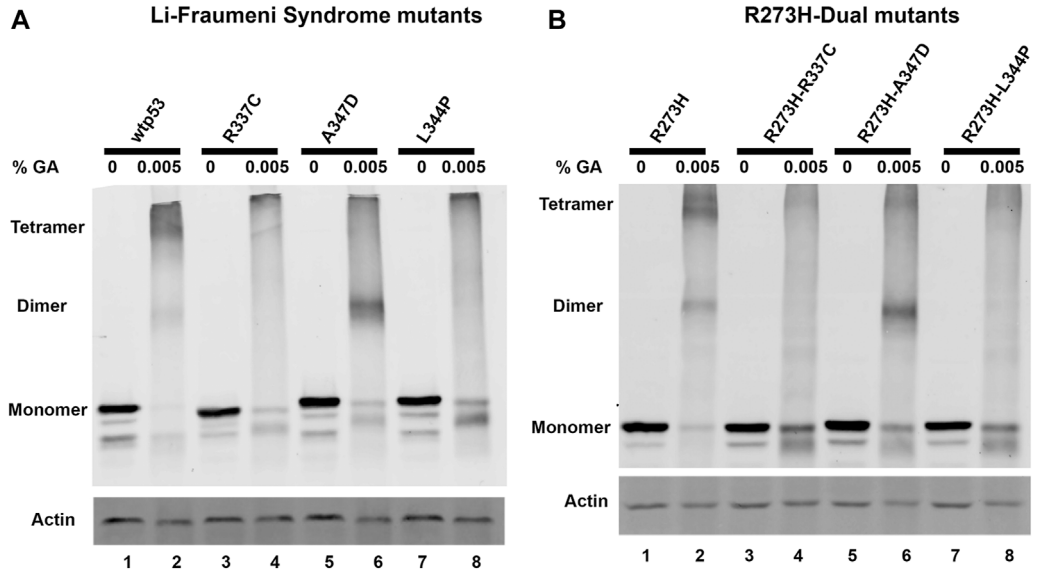

FIGURE 1 | R273H-dual mtp53 mutants destabilize oligomerization of mtp53 R273H similar to Li-Fraumeni Syndrome single mutants. HCT116 p53−/− cells were transiently transfected with plasmids expressing either **(A)** wtp53, or single mutants R337C, A347D or L344P; or **(B)** mtp53 R273H, or dual mutants R273H-R337C, R273H-A347D, or R273H-L344P. After 24 h post transfection, cells were pelleted, and lysate prepared. 50 μg of the resulting cell lysates were treated with either 0% (lanes 1,3,5,7) or 0.005% (lanes 2,4,6,8) glutaraldehyde for 20 min at room temperature. Samples were run on an 8% SDS-PAGE and oligomerization determined by western blotting using anti-p53 DO1 antibody. Actin was used as a normalizer and showed very minor shift in mobility with 0.005% glutaraldehyde. Data presented was reproduced in three biological replicates.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 9. Results are expressed as mean +SEM. Statistical significance for hypothesis testing was performed by a one-way ANOVA with multiple comparison. The following format was used to assign significance based on *p*-value: **** represents a *p*-value ≤ 0.0001 and ns represent non-significant.

RESULTS

p53 Tetramer Formation Is Destabilized by Introducing Mutations in the OD

We used site-directed mutagenesis to introduce oligomerization mutations to change p53 amino acid residues R337C, A347D, or L344P in plasmids for exogenous expression of either wtp53 or mtp53 R273H. When the Li-Fraumeni OD-specific associated point mutations were introduced individually, or as dual mutants within mtp53 R273H, they destabilized tetramer formation (**Figures 1A,B**). The exogenously expressed wtp53 and mtp53 R273H proteins predominantly formed dimers and tetramers when 0.005% glutaraldehyde was added (**Figures 1A,B**, lanes 1, 2), whereas both single A347D and dual R273H-A347D polypeptides shifted to form predominantly dimers (**Figures 1A,B**, lanes 5, 6). The Li-Fraumeni mutations R337C and L344P, as well as the dual mutants R273H-R337C and R273H-L344P, were unable to form dimers or tetramers (**Figures 1A,B**, lanes 3–8). We also confirmed that endogenous mtp53 in many different human breast cancer cell lines formed tetramers (**Supplementary Figure S1**). These data indicate that mtp53 can exist as tetramers in human cells, and that we successfully disrupted mtp53 R273H oligomerization in the R273H R337C, A347D, or L344P dual mutants.

Transactivation of *p21* and *MDM2* Is Activated by wtp53, but Not mtp53 R273H, or OD Mutants

The p53 protein activates the transcription of the cyclin-dependent kinase *p21* and the p53 inhibitor *MDM2* (Levine, 1997; Wu and Levine, 1997). We wanted to confirm that destabilization of wtp53 oligomerization blocked transactivation. We expressed the R337C, A347D, or L344P single or dual mtp53 R273H in HCT116 p53−/− cells and observed significant *TP53* message and protein expression in all the transfection experiments (**Figures 2A,B**, compare empty vector (EV) to expression constructs as indicated). As expected, upregulation of the endogenous targets *p21* and *MDM2* occurred in the presence of wtp53 expression (**Figures 2C,D**), and the LFS mutations blocked the ability of p53 to transactivate *p21* and *MDM2* (**Figures 2C,D**). Equally as predictable, the mtp53 R273H mtp53 expression was unable to activate expression of p*21* or *MDM2*, and dual mutants were no different (**Figures 2C,D**). The mtp53 R273H has been shown to activate transcriptional targets *CDC7* and *RRM2* (Kollareddy et al., 2015; Datta et al., 2017). As such, we examined the amount of mRNA and protein expression levels of these targets. We examined whether mtp53 R273H, or the mtp53 R273H dual mutants, significantly upregulated the expression of either *CDC7* or *RRM2* (**Supplementary Figure S2**). However, we did not detect upregulation for these genes. This

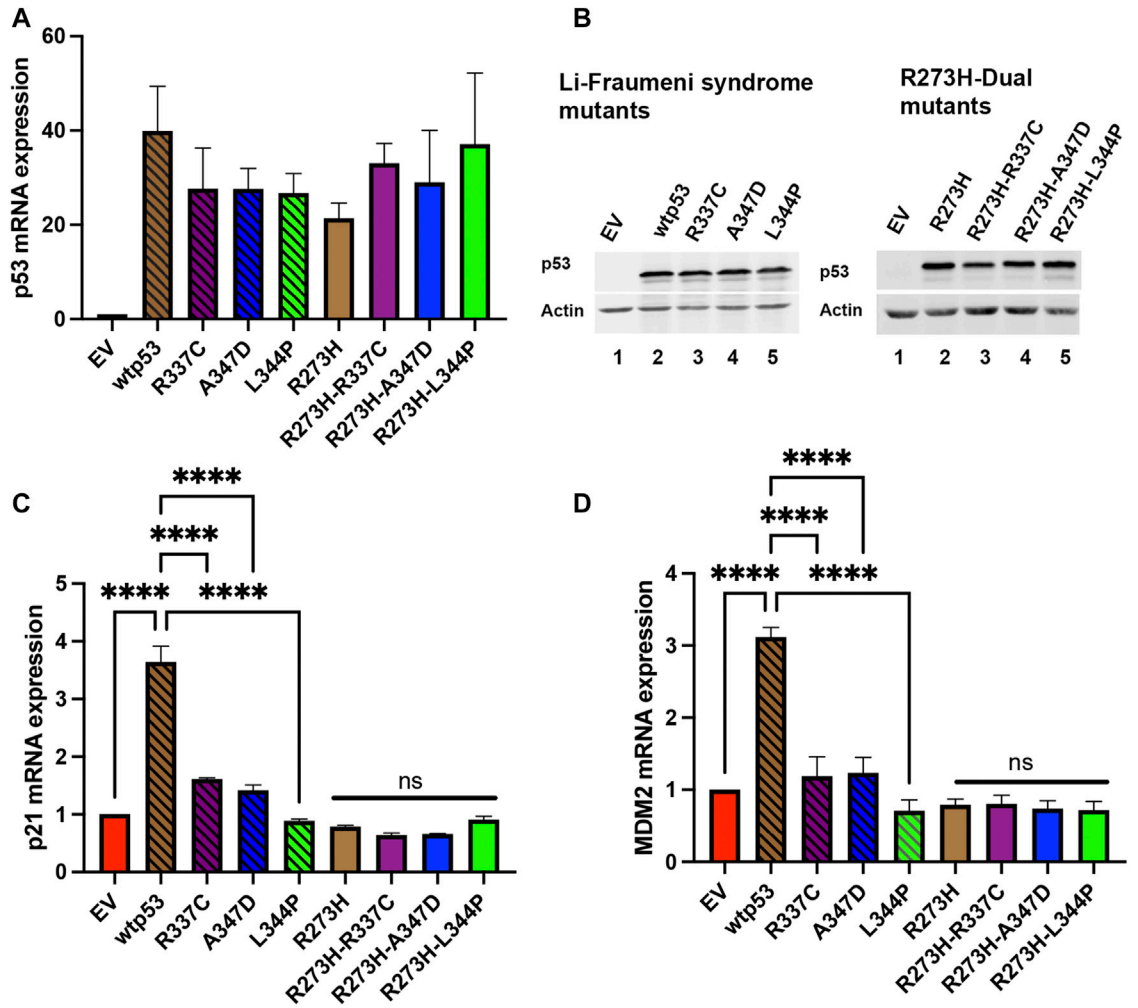

FIGURE 2 | Both *p21* and *MDM2* are activated by wtp53 but not mtp53. HCT116 p53–/– cells transfected with plasmids expressing wtp53, or single mutants R337C, A347D or L344P; or mtp53 R273H, or dual mutants R273H-R337C, R273H-A347D, or R273H-L344P were harvested 24 h post-transfection. The pellets were divided into two and used for either protein or RNA extraction. RNA was extracted from pellet and 5 µg of RNA used for cDNA synthesis. The TaqMan real-time PCR was used to measure the mRNA expression levels of *p53* **(A)**, *p21* **(C)** and *MDM2* **(D)** target genes using *GAPDH* as endogenous control. The data represent an average of three independent biological replicates. A one-way ANOVA was used to determine the statistical significance of the data. The following format was used to assign significance based on *p*-value: **** represents a *p*-value ≤ 0.0001 and ns represent non-significant. **(B)** Pellets were lysed in RIPA buffer and 25 µg of lysate loaded on a 10% SDS-PAGE and probed with anti-p53 DO1 antibody. Actin was used as a normalizer. Data represent three independent biological replicates.

could be due to low transfection efficiency (30% efficiency), or other variables that may have influenced mtp53 mediated transcription effects.

Destabilizing Oligomerization of mtp53 Does Not Block mtp53 Interaction With Chromatin

Endogenous mtp53 R273H, and other mtp53 proteins, tightly tether to chromatin (Polotskaia et al., 2015; Qiu et al., 2017). We tested whether the tetramerization state of mtp53 was crucial for the mtp53 chromatin association. We transiently transfected in mtp53 R273H without, and with, the OD mutations. To further examine how dual mutants influenced chromatin interaction, we treated cells with a combination of the alkylating agent temozolomide (Temo) and the PARP1 trapping drug talazoparib (Tal) to induce replication stress and trap PARP1 on the chromatin (Qiu et al., 2017; Xiao et al., 2020). We then isolated chromatin by either chromatin fractionation, or the more stringent detergent insoluble fractionation assay, as methods to assess protein-DNA tethering. We observed mtp53 R273H and mtp53 R273H-dual oligomerization mutants tightly tethered to chromatin (**Figures 3A,B**, with imageJ quantification shown in **Supplementary Figures S3A,B**). The replication stress did not significantly alter the ability of mtp53 to interact with chromatin regardless of the OD domain status (**Figures 3A,B**; **Supplementary Figures S3A,B**). We therefore conclude that oligomerization of mtp53 R273H is not required for mtp53 chromatin association. Moreover, both PARP and MCM2 interacted well with the chromatin in both unstressed and

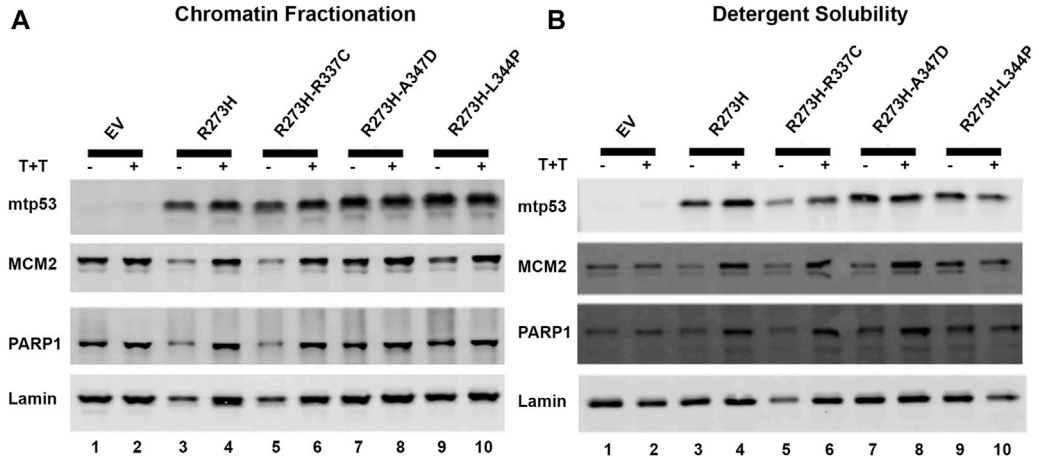

FIGURE 3 | Destabilizing oligomerization of mtp53 R273H does not block the interaction with chromatin. The chromatin fractionation and the detergent solubility assays were used to fractionate lysates prepared from HCT116 p53–/– cells transfected with mtp53 R273H, or dual mutants R273H-R337C, R273H-A347D, or R273H-L344P into cytosolic/soluble fraction and chromatin/insoluble fraction. Analysis of lysates from cells without drug treatment (lanes 1,3,5,7,9) or with a combination of temozolomide (T) at 1 mmol/L plus talazoparib (T) at 10 μmol/L for 4 h (lanes 2,4,6,8,10) was carried out. Binding of mtp53, MCM2, and PARP1 to chromatin isolated from partially purified lysed nuclei (chromatin fractionation assay) or high detergent total cell lysates (detergent solubility assay) was assessed by western blot analysis. 25 μg of chromatin protein was loaded on 10% SDS-PAGE gel. **(A)** Western blot depicting the chromatin association of p53, MCM2, and PARP1 after chromatin fractionation. **(B)** Western blot analysis depicting the chromatin association of p53, MCM2, and PARP1 after detergent solubility. The protein expression level of each target protein was normalized to lamin. The data represent an average of three independent biological replicates. ImageJ quantification of protein expression level is represented in **Supplementary Figures S3A,B**.

stressed conditions. The p53 protein that cannot form tetramers does not activate gene transcription. However, the possibility exists that monomers and dimers are able to maintain non-specific chromatin interactions. We compared wtp53, with and without OD mutations, for their general chromatin interactions by transfecting HCT116 p53 –/– cells with plasmids expressing wtp53 or the single OD mutants. Western blot analysis of the chromatin fractionated samples demonstrated that the different oligomerization forms tethered well to the chromatin. As such, mutation in the OD of p53 disrupts transcription factor function but does not disrupt the ability of the protein to generally interact with chromatin (**Supplementary Figure S3C**).

Destabilizing Oligomerization of R273H mtp53 Does Not Inhibit Interaction Between mtp53 and MCM2

We tested if oligomerization influenced the interaction of mtp53 R273H with either MCM2 or PARP1 by using the proximity ligation assay (PLA), which was previously used to demonstrate their endogenous interactions (Xiao et al., 2020). We used transfected HCT116 p53–/– cells in order to compare the interactions with similar levels of exogenously expressed protein for wtp53 and R273H mtp53 single and dual mutants. The expression of GFP was used as a marker for cells expressing p53 and importantly PLA foci were only detected in p53 expressing cells. Previously we had detected a low number of PLA foci for wtp53 in an endogenously expressing cell line. This was due to the fact that endogenous wtp53 protein is maintained at low levels in MCF7 cancer cells due to degradation by the E3 ubiqutin ligase MDM2 (Xiao et al., 2020). Interestingly, when the expression level was the same we detected similar interaction levels between MCM2 with both wtp53 and mtp53 R273H (**Figures 4A,B**). In contrast, the dual mtp53 R273H-OD mutants demonstrated slightly more MCM2-mtp53 associated foci (**Figures 4A,B**). The interaction between mtp53 and PARP1 was also maintained after introducing mutations to destabilize tetramer formation, with no observable increase for the dual mutants (**Figures 4C,D**). This suggests that R273H hotspot mtp53 may interact with replicating DNA in forms that are not tetrameric. We also assessed the single-OD mutants and observed interactions between the single-OD p53 mutants and MCM2 as well as PARP1 (**Supplementary Figure S4**). This further supports the possibility that non-tetramerized p53 may function in alternative, non-transcription related, pathways that mediate GOF activity.

Deletion of the Non-specific DNA Binding Domain of mtp53 Decreases the Interaction Between mtp53 R273H and MCM2

Generation of R273H mtp53 deletions of a small portion of the C-terminus (R273HΔ381-388) and a larger deletion removing some of the OD and all of the C-terminus (R273HΔ347-393) in MDA-MB-468 cells causes replication stress (Ellison et al., 2021). The larger deletion R273HΔ347-393 dual mutant causes drastic inhibition of cell proliferation. As such, we wondered if the entire deletion of the C-terminal non-specific DNA binding domain R273HΔ347-393 inhibited the interaction of mtp53 R273H with the replication machinery. We tested the interaction between mtp53 and MCM2 using the PLA method and observed that R273HΔ347-393, but not the smaller deletion R273HΔ347-393,

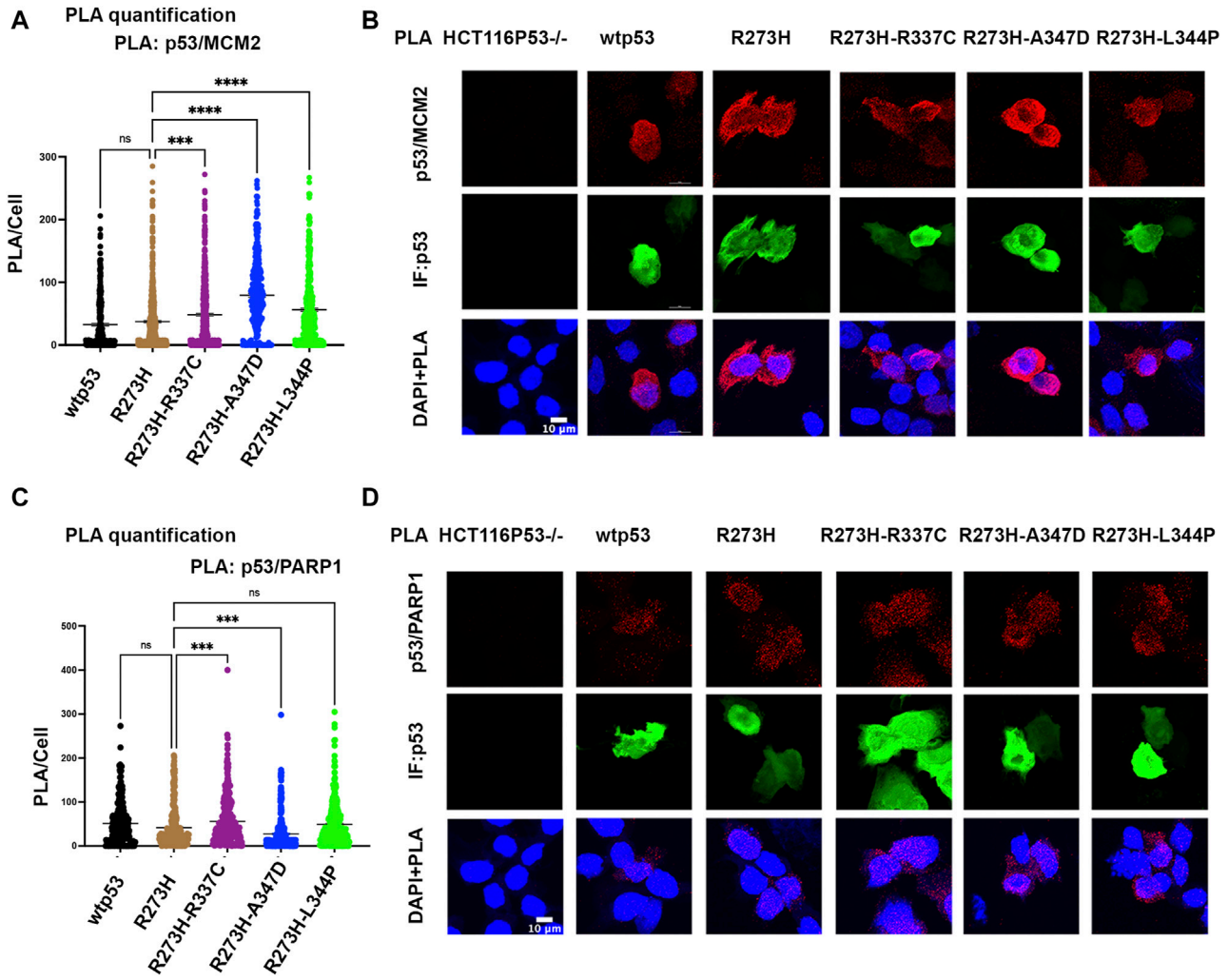

FIGURE 4 | Destabilizing oligomerization of R273H mtp53 does not block the interaction between mtp53 and MCM2. HCT116 p53–/– cells were transfected with wtp53, mtp53 R273H, or dual mutants R273H-R337C, R273H-A347D, R273H-L344P expressing plasmids. **(A)** Analysis of p53/MCM2 complexes by *in situ* proximity ligation assay (PLA). Fluorescent foci per cell were counted using Cellprofiler software and depicted as a scatter plot using GraphPad Prism 9. The data represent a scatter plot with n = 3. An ordinary one-way ANOVA was used to determine the statistical significance of the data. The following format was used to assign significance based on *p*-value: **** represents a *p*-value ≤ 0.0001 and ns represent non-significant. **(B)** Representative confocal microscope images of p53/MCM2 complexes (red) by *in situ* proximity ligation assay (PLA), p53 expression (green) by immunofluorescence microscopy. DNA was counterstained with DAPI (blue). The z-stack maximum intensity projection images are shown. Three independent experiments were performed. (Scale bar = 10 µm). **(C)** Analysis of p53/PARP1 complexes by *in situ* proximity ligation assay (PLA). Fluorescent foci per cell were counted using Cellprofiler software and depicted as a scatter plot using GraphPad Prism 9. The data represent a scatter plot with n = 3. An ordinary one-way ANOVA was used to determine the statistical significance of the data. The following format was used to assign significance based on *p*-value: **** represents a *p*-value ≤ 0.0001 and ns represent non-significant. **(D)** Representative confocal microscope images of p53/PARP1 foci/complexes (red) by *in situ* proximity ligation assay (PLA), p53 expression (green) by immunofluorescence microscopy. DNA was counterstained with DAPI (blue). The z-stack maximum intensity projection images are shown. Three independent experiments were performed. (Scale bar = 10 µm).

exhibited decreased interaction with the MCM2 replication helicase (**Figures 5A,B**).

DISCUSSION

Wild-type p53 requires tetramization to be a functional transcription factor but oncogenic mtp53 does not act as a direct transcription factor. As such, it remains to be determined what roles are played by the different oligomerization forms of oncogenic mutant p53. The newly generated dual mutants have both a DNA binding domain mutation and an oligomerization domain mutation. Dual mutants are not naturally occurring in cancers. Mutations in the OD that disrupt p53 transcription factor function may allow for p53 functions (that to date have not been discovered and/or described) to be co-opted in the mutant p53 isoforms. It is possible that monomers of p53 may have a transcription-independent chromatin associated function, and our work may provide clues for how equilibrium between different p53

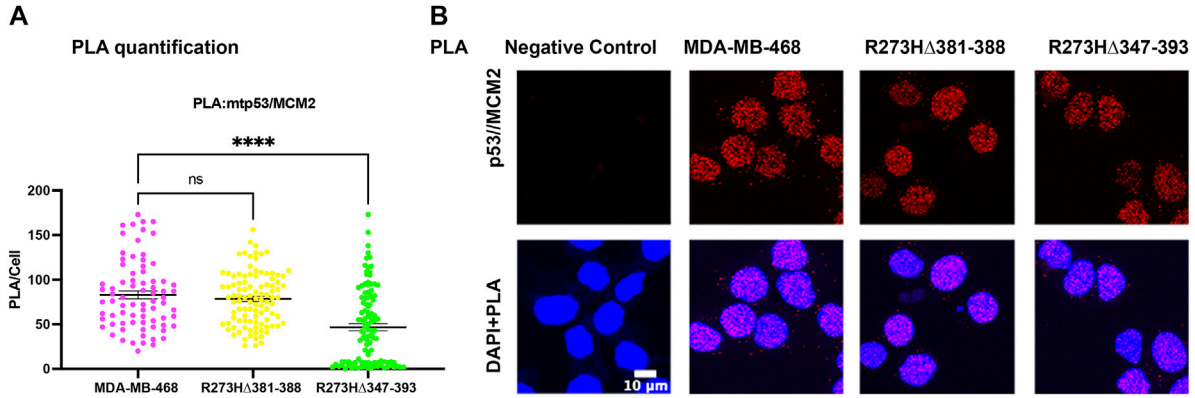

FIGURE 5 | Endogenous deletions in the OD of R273H mtp53 decreases the interaction between mtp53 and MCM2. **(A)** Analysis of p53/MCM2 complexes by *in situ* proximity ligation assay (PLA) in MDA-MB-468, R273HΔ381-388 and R273HΔ347-393 cells. Fluorescent foci per cell were counted by Cellprofiler software and depicted by a scatter plot using GraphPad Prism 9. The data represent a scatter plot with n = 3. An ordinary one-way ANOVA was used to determine the statistical significance of the data. The following format was used to assign significance based on *p*-value: **** represents a *p*-value ≤ 0.0001 and ns represent non-significant. **(B)** Representative confocal microscope images of p53/MCM2 foci/complexes (red) by *in situ* proximity ligation assay (PLA) in MDA-MB-468, R273HΔ381-388 and R273HΔ347-393 cells. DNA was counterstained with DAPI (blue). The z-stack maximum intensity projection images are shown. Three independent experiments were performed. (Scale bar = 10 μm).

oligomerization forms influence different cellular functions. The observation of altered tumor-suppressor function resulting from LFS mutations within the OD is similar to that observed in hotspot DNA binding domain mutants (Levine, 2021). Both types of mutations lead to a loss of function for wtp53 tumor suppressor functions. In the resulting cancers high levels of stable mtp53 occur (Kim and Lozano, 2018). We have explored how dual region mutations, in both the DNA binding domain and OD, influence the mtp53 replication-associated chromatin functions. We were able to change the oligomerization state of mtp53 R273H such that the R273H-dual mutants possessed similar oligomerization to their corresponding single LFS mutants (**Figure 1**).

The LFS mutations R337C, A347D, and L344P within the OD as expected blocked the transactivation of wtp53 (**Figure 2**). The DNA binding domain mutation R273H compromised the ability of the protein to activate transcription from endogenous p53 responsive elements. In the case of the mtp53 R273H-dual mutants, the presence of an extra mutation that prevented tetramer formation, not surprisingly, had no influence on the transactivation ability (**Figure 2**). We did not observe mtp53 R273H mediated transactivation of either *CDC7* or *RRM2* (**Supplementary Figure S2**). This supports the recent finding that outcomes on activation of new genes may result from aneuploidy and are not direct results of mtp53 R273H transactivation (Redman-Rivera et al., 2021).

Chromatin tethering of mtp53 has implications in DNA replication and repair mechanisms. Prior to this study, various reports confirmed that most of the chromatin association of wtp53 occurs as a tetramer (McLure and Lee, 1998; McLure and Lee, 1999; Weinberg et al., 2004). We observed that hotspot GOF mtp53 R273H can form tetramers in cancer cell lines (**Supplementary Figure S1**) but that tetramerization is not required for chromatin interaction, or interaction with MCM2 (**Figures 3**, **4**). The DNA replication machinery is under the regulation of protein complexes that control origin licensing, firing, unwinding, and relaxation (Klusmann et al., 2016). GOF mtp53 has been implicated in a variety of DNA replication processes, including an increase in replication origin firing and enabling the interaction between TopBP1 with Treslin to induce Cdk2 (Kollareddy et al., 2015; Singh et al., 2017). We previously reported a close association between mtp53, replicating DNA, and PARP1 (Xiao et al., 2020). Herein, we explored whether the oligomerization of mtp53 R273H was required for it to interact with PARP1, and the replication helicase MCM2. Our results suggest that the interaction between mtp53 R273H and PARP is not significantly altered by destabilizing mtp53 tetramer formation. On the other hand, there was a slight increase in the interaction between mtp53 R273H-dual OD mutants and MCM2 (**Figure 4**). This suggests that non-tetrameric forms of p53 may interact more often with the DNA replication machinery. Interestingly, the absence of the entire C-terminal domain (which is involved in non-specific DNA binding and nuclear localization) in the R273HΔ347-393 mutant (Ellison et al., 2021), had a reduced interaction with MCM2 (**Figure 5**). Taken together, the data presented here showed that oncogenic mtp53 R273H can form tetramers, but that the dynamics of tetramer formation and the C-terminal non-specific DNA binding domain may differentially regulate the GOF replication-associated activities. We are in the process of carrying out further experiments to explore how destabilizing tetramer formation and non-specific DNA binding influence the association between mtp53 R273H and replicating DNA.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

Author Contributions: Conception and design: JB. Development and methodology: GA, NE, JB. Acquisition of data: GA, NE, DL, DC, and VE. Analysis and interpretation of data: GA, NE, DC, GX, VE, and JB. Writing, review, and or revision of the manuscript: GA, NE, DL, DC, GX, VE, and JB. Study supervision: JB.

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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.2021.772315/full#supplementary-material

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REVIEW
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The Basally Expressed p53-Mediated Homeostatic Function

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Apart from mutations in the *p53* gene, p53 functions can be alternatively compromised by a decrease in nuclear p53 protein levels or activities. In accordance, enhanced p53 protein turnover due to elevated expression of the critical p53 E3 ligase MDM2 or MDM2/MDMX is found in many human cancers. Likewise, the HPV viral E6 protein-mediated p53 degradation critically contributes to the tumorigenesis of cervical cancer. In addition, growth-promoting signaling-induced cell proliferation is accompanied by p53 downregulation. Animal studies have also shown that loss of p53 is essential for oncogenes to drive malignant transformation. The close association between p53 downregulation and carcinogenesis implicates a critical role of basally expressed p53. In accordance, available evidence indicates that a reduced level of basal p53 is usually associated with disruption of homeostasis, suggesting a homeostatic function mediated by basal p53. However, basally expressed p53 under non-stress conditions is maintained at a relatively low abundance with little transcriptional activity, raising the question of how basal p53 could protect homeostasis. In this review, we summarize the findings pertinent to basal p53-mediated activities in the hope of developing a model in which basally expressed p53 functions as a barrier to anabolic metabolism to preserve homeostasis. Future investigation is necessary to characterize basal p53 functionally and to obtain an improved understanding of p53 homeostatic function, which would offer novel insight into the role of p53 in tumor suppression.

Keywords: basal p53, homeostasis, metabolism, tumor suppression, p53-mediated barrier

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INTRODUCTION

The function of p53 is universally disrupted in human cancers, either by a mutation in the *p53* gene locus or aberration in p53 regulation (Levine, 2020). Approximately 50% of all human cancers lost p53 function due to gene mutations, which occur primarily within the p53 DNA binding domain, underscoring the necessity of p53 binding to DNA for its tumor suppressor function (Vousden and Lane, 2007). In this context, p53 is best characterized as a transcription factor. Upon activation, p53 induces the expression of a host of genes that govern diverse cellular processes such as cell cycle progression, apoptosis, cell differentiation, and senescence, among others (Vousden and Prives, 2009), including regulation of cellular metabolic pathways. For example, studies have revealed that p53 can stimulate the mitochondrial TCA cycle by inducing the expression of SCO2 (synthesis of cytochrome oxidase 2), a critical regulator of the cytochrome c oxidase complex (Matoba et al., 2006), whereas suppressing glycolysis by repressing the expression of glucose transporters 1 and 4 (GLUT-1 & 4) (Schwartzenberg-Bar-Yoseph et al., 2004). In addition, p53 can transcriptionally induce the expression of the fructose-2,6-bisphosphatase TIGAR (p53 induced glycolysis and apoptosis regulator) (Bensaad et al., 2006). Together, available information indicates that p53 directs

cellular metabolism away from glycolysis and towards oxidative phosphorylation (Vousden and Ryan, 2009). However, recent studies revealed that many p53 transcription-mediated canonic activities are dispensable for its tumor suppression (Kastenhuber and Lowe, 2017). In addition, acute DNA damage-induced p53 transcriptional activity was also found expendable for inhibition of carcinogenesis (Christophorou et al., 2006). Thus, the available information suggests that there might be unrecognized activities mediated by p53 DNA binding critical for tumor suppression.

Apart from *p53* gene mutations, the activity of p53 can be attenuated by a reduction in nuclear p53 levels (Kastenhuber and Lowe, 2017). Indeed, diminished nuclear p53 protein abundance due to overexpression of the critical p53 E3 ligase MDM2 or MDM2/MDMX is found in many human cancers (Karni-Schmidt et al., 2016). Likewise, the HPV viral E6 protein-mediated p53 degradation critically contributes to the development of cervical cancer (Scheffner et al., 1990). Thus, preclinic as well as clinical studies suggest that the amount of basally expressed nuclear p53 and its DNA sequence-specific binding are critical for p53's tumor-suppressive function.

THE REGULATION OF P53

Because of its growth inhibitory activity, p53 is normally maintained at a relatively low level under physiological conditions (Vousden and Prives, 2009). Ample evidence indicates that p53 is primarily regulated, mainly at the post-translation level via protein turnover. Among many proteins involved in the regulation of p53 turnover, MDM2 stands out as the dominant E3 ligase specifically targeting p53 for ubiquitination and subsequent proteasome-dependent degradation (Haupt et al., 1997; Ringshausen et al., 2006). While MDMX, the structural homolog of MDM2, lacks intrinsic E3 ligase activity, it can modulate MDM2 E3 ligase activity via forming the MDM2/MDMX complex (Linares et al., 2003; Kawai et al., 2007). Genetic studies have provided convincing evidence demonstrating that MDM2 and MDMX are two essential negative regulators of p53, and the formation of the MDM2/MDMX complex appears crucial in p53 control (Parant et al., 2001; Huang et al., 2011). p53, MDMX, and MDM2 form a highly dynamic regulatory core that comprises positive and negative feedback loops' which ensure tight regulation of p53 in non-stress conditions and its swift response to stress conditions (Gu et al., 2002). As the critical upstream modulator of p53, the MDM2/MDMX complex integrates myriad intrinsic and external signals to regulate p53 response to the perturbation of homeostasis (Wade et al., 2013). In line with a protein containing the nuclear localization sequence (NLS), MDM2 is primarily nuclear-localized. Of note, despite sharing a high degree of structural similarity with MDM2, MDMX lacks the NLS and is a predominantly cytoplasmic protein (Gu et al., 2002). MDMX, however, can translocate into the nucleus upon binding to and forming a complex with MDM2. Given its cytoplasmic distribution, it is conceivable that MDMX serves as the sentinel for various signaling cues directed towards the MDM2/MDMX complex and aimed at either suppressing or activating p53 (Shadfan et al., 2012; Wang et al., 2020). Studies have shown that in response to growth-promoting signals, many mitogenic protein kinases can inhibit p53 activation via enhancing MDM2/MDMX stability and, specifically, through post-translational modifications of MDMX (Lopez-Pajares et al., 2008; Gerarduzzi et al., 2016).

Apart from MDM2, several additional E3 ligases were reported to promote p53 for ubiquitinate/proteasome degradation, including Pirh2, Cop1, TRIM proteins CHIP, RBCK1, and ARF-BP1, among others (Sane and Rezvani, 2017). Evidence suggests that while important, these E3 ligases may regulate p53 turnover in a context-dependent manner. For instance, Cop1 is amplified in certain human cancers such as hepatocellular carcinomas and breast cancer where p53 is not frequently mutated, suggesting an essential role of Cop1 in p53 inhibition in the context of these types of human cancers.

In line with the general feature of the two-directional reaction in protein post-translational modifications, p53 ubiquitination can be reversed by deubiquitination, a reaction commonly catalyzed by ubiquitin-specific proteases (USPs) (Kwon et al., 2017). Among several USPs that are known to target p53, USP7 or HAUSP is one of the relatively well-characterized USPs that critically contribute to the regulation of p53 stability (Lim et al., 2004). Of interest is that HAUSP can also target MDM2 for deubiquitination representing a complex mechanism of p53 regulation (Li et al., 2004). Ubiquitination of p53 is often associated with its nuclear export to the cytoplasm (Boyd et al., 2000), where the ubiquitinated p53 is recognized as a substrate for degradation by proteasome resulting in a decrease in p53 abundance. However, under certain conditions where the proteasome activity is hampered, p53 may accumulate in the cytoplasm.

Nonetheless, p53 cytoplasmic distribution prevents it from binding to DNA, equivalent to functional p53 inactivation. Indeed, cytoplasmic p53 accumulation is found in a subset of human cancers (Lu et al., 2000). While the proteasome is primarily the place for p53 protein turnover, the autophagy-lysosome machinery has also been reported to participate in regulating p53 levels. However, the contribution of the autophagy-lysosome axis to p53 degradation seems to limit mutant p53 in a context-dependent manner (Xu et al., 2021).

In addition to the change in the p53 protein abundance, p53 activity can also be regulated via post-translational modifications. For example, by counteracting against p53 acetylation, which is necessary for its transcription activity, deacetylation of p53 by HDAC such as histone deacetylase eight diminishes p53 transcription activity (Qi et al., 2015). Another type of post-translational modification is protein methylation, which was also reported to be one of the mechanisms of p53 regulation. For instance, histone lysine methyltransferases KMT5 (Set9), KMT3C (Smyd2), and KMT5A (Set8) were reported to methylate p53 at specific C-terminal lysine residues. Thus, dependent on the site of modification, p53 methylation can either augment or attenuate p53 transcriptional activity. Furthermore, like acetylation/deacetylation, methylated lysine can be demethylated by the lysine-specific demethylase, such as KDM1 (LSD1), promoting p53 demethylation in interfering with the interaction of p53 with

its co-activator 53BP1 and subsequent the induction of apoptosis (Scoumanne and Chen, 2008).

THE P53-MEDIATE BARRIER TO CELL PROLIFERATION

It is well documented that p53 is typically growth inhibitory, or p53 is usually incompatible with increased cell proliferation. Based on this notion, induction of cell proliferation would predict a reduction in p53 activity/level. Zwang et al. reported that mitogen such as EGF-induced proliferation in normal human mammary epithelial cells was mediated by two temporally separable waves of growth signals during which induction of metabolic pathways is associated with downregulation of antiproliferative genes (Zwang et al., 2011). Many of the antiproliferative genes are the target genes of p53. Of interest is the finding that the second wave of growth signal drives cells passing the restriction point concurrent with p53 downregulation. To demonstrate the importance of p53, the authors used siRNA to knockdown p53 expression. Remarkably, p53 downregulation allowed cells to bypass the second wave of growth signals to cross the restriction point entering the S-phase. The study revealed that growth factor-induced cell proliferation must override a p53-dependent constraint, consistent with the notion that p53 functions as a barrier to cell proliferation (Vousden and Lane, 2007). Though the mechanism of EGF-induced p53 downregulation was not investigated, the authors showed that activation of PI3K and AKT was necessary to reduce the expression of p53-mediated antiproliferative genes. It has been shown that AKT can phosphorylate both MDM2 and MDMX, resulting in enhanced p53 ubiquitination/degradation, providing a plausible mechanism underlying the grow-promoting signal-induced p53 downregulation (Lopez-Pajares et al., 2008). In line with Zwang et al., Lei et al. also reported p53 downregulation in mitogen-induced cell proliferation (Lei et al., 2011; Zwang et al., 2011). The studies together implicate an essential role of basally expressed p53 in restraining cell proliferation. Pro-growth signals breach this growth constraint by stimulating MDM2/MDMX-mediated p53 turnover, promoting cell proliferation.

In agreement with the fundamental importance of metabolism in cell growth, induction of cell proliferation is contingent upon metabolic reprogramming from catabolic to anabolic metabolism. In accordance with its function in growth inhibition, p53 typically antagonizes anabolic pathways while stimulating oxidative phosphorylation. Available information indicates that p53 mainly regulates cellular metabolism in a transcription-dependent fashion (Vousden and Ryan, 2009). The basal p53-mediated restraint on cell proliferation would suggest a scenario in which basally expressed p53 could keep anabolic metabolism in check under the homeostatic condition. However, basal p53 typically possesses little transcription activity. Therefore, it is largely unknown whether and how p53 could regulate metabolism independent of its transactivation activity. An early study by Kawauchi et al. showed that loss of p53 either via gene knockout or siRNA-mediated knockdown was associated with induction of glycolysis (Kawauchi et al., 2008). Mechanistically, the authors demonstrated that p53 loss resulted in activation of NF-κB, which induced the expression of Glut3, promoting glycolytic metabolism. While the antagonistic interaction between p53 and NF-kB has been well documented, the study by Kawauchi et al. implicates that basal p53 can keep the NF-kB pathway under control, and a mere drop of p53 level would unrestraint its restriction unleashing NF-kB activity to promote anabolic metabolism (Kawauchi et al., 2009). Of note, Zwang et al. also reported that p53 downregulation was associated with induction of metabolic enzymes related to steroid, cholesterol, and lipid metabolism, whose intermediate products are critical substrates for cell division (Zwang et al., 2011).

The studies together suggest a model in which basally expressed p53 can keep anabolic metabolism in check to maintain homeostasis. In accordance, cell growth signals disable this p53-mediated metabolic constraint to induce anabolic metabolism, promoting cell proliferation. Therefore, further investigation is warranted to explore how basally expressed p53 keeps anabolic pathways under control.

P53-MEDIATE HOMEOSTATIC REGULATION OF IMMUNE AND INFLAMMATORY RESPONSE

Like cell proliferation that depends on anabolic metabolism, T cell activation represents another typical process involving a metabolic switch from catabolic to anabolic metabolism. Wang et al. demonstrated that metabolic reprogramming from the TCA cycle to the anabolic pathways, including glycolysis, pentose-phosphate, and glutaminolysis, is coupled with T cell activation (Wang et al., 2011). This switch to anabolic metabolism is necessary to meet the increased demands for the bioenergetic and biosynthesis as suppression of anabolic pathways genetically or pharmacologically blocked T cell activation. Mechanistic analysis revealed that the master transcription factor Myc is responsible for the increased glycolysis and glutaminolysis. Remarkably, a study by Watanabe et al. revealed that p53 downregulation is necessary for antigen-specific activation of T cell proliferation (Watanabe et al., 2014). While the authors did not examine the metabolic changes, increased, T cell proliferation is expected to be concurrent with metabolic reprogramming, which many studies have validated since the report by Wang et al. (Wang et al., 2011). Hence, it is conceivable to speculate that p53 downregulation enables the metabolic switch to anabolism to fuel T-cell proliferation, implicating an antagonistic interaction between p53 and Myc in the regulation of metabolism.

Studies have also uncovered an important role of p53 in B cell activation and expansion (Phan and Dalla-Favera, 2004). During the germinal center (GC) reaction in the lymph nodes, the activated B cells undergo cycles of expansion and specific genome remodeling, for instance, somatic hypermutations and class switch recombination. Highly expressed BCL6 in B cells within the GC is essential to regulate these events. BCL6

transcriptionally represses p53 expression (Phan and Dalla-Favera, 2004), which not only evades p53-dependent apoptosis but also allows B cell proliferation/expansion. Like T cells, B cell proliferation also relies on metabolic reprogramming, where mTOR and c-Myc-mediated glycolysis and anabolic metabolism were reported to contribute to B cell activation in the GC (Calado et al., 2012; Dominguez-Sola et al., 2012; Ersching et al., 2017). While the studies did not directly examine the interaction between p53 and c-Myc/mTOR, it is conceivable that p53 downregulation is conducive to the stimulation of c-Myc/mTOR (Feng et al., 2005). Further studies are warranted to address the antagonistic interactions.

The inflammatory response is energy-consuming process and relies on anabolic programs. For instance, in response to LPS stimulation, macrophages undergo metabolic reprogramming from oxidative phosphorylation to glycolysis via activation of the mTOR-HIF1α pathway (Covarrubias et al., 2015), resulting in induction of μPFK2 (Rodríguez-Prados et al., 2010) and GLUT1 (Freemerman et al., 2014). The production of IL1β is also contingent upon the activation of mTOR-HIF1α (Tannahill et al., 2013; Moon et al., 2015a) and fatty acid synthase (Moon et al., 2015b). The importance of p53 in inflammation was revealed with p53 knockout mice that exhibited inflammation so severe that some of the mice died from unresolved inflammation before the onset of tumorigenesis (Martínez-Cruz et al., 2009). Such a role of p53 in inflammation seems not unexpected considering the tight association of chronic inflammation with tumorigenesis (Gudkov and Komarova, 2016), though the underlying molecular details are still being actively investigated.

Macrophage is one of the major cell types that contribute to the inflammatory responses. Depending on stimuli, macrophages can be induced into different functional states, for instance, M1 or classically activated macrophages and M2 or activated macrophages, according to the simplified classification method. M1 macrophages are pro-inflammatory that is characterized by the release of inflammatory cytokines [IL-1β, IL-12, IL-6, and tumor necrosis factor (TNF)], reactive oxygen species (ROS), and nitrogen species, whereas M2 macrophages, in contrast, participate in the anti-inflammatory response to facilitate wound healing and tissue repair. Importantly, M1 and M2 are intimately linked to and controlled by distinct metabolic programs (Covarrubias et al., 2015). Stimulation of M1 polarization is associated with induction of glycolysis, fatty acid synthesis, amino acid metabolism, and inflammatory cytokines. The transcriptional program in M1 macrophage is primarily mediated by the mTOR-HIF-1α pathway (Covarrubias et al., 2015). M2 macrophages preferentially rely on β-oxidation of fatty acids and mitochondrial respiration for their sustenance and functional activation. Type 2 cytokines, such as IL-4 and IL-13, signal to activate the latent STAT6 transcription factor through their cognate receptors. STAT6 promotes the metabolic transition to oxidative metabolism by inducing genes essential in FAO and mitochondrial biogenesis. In addition, STAT6 transcriptionally induces PGC-1β, PPARγ, and PPARδ, which synergize with STAT6 to enhance the expression of alternative activation markers and stabilize the metabolic switch to oxidative metabolism.

In support of the role of p53 in inflammation, Li et al. reported that loss of p53 stimulated whereas activated p53 impeded M2 macrophage polarization (Li et al., 2015). Using a combination of genetic and pharmacological approaches, the authors demonstrated that p53 selectively inhibits M2 polarization by downregulating M2 gene expression. While the authors did not examine the metabolic changes associated with macrophage polarization, they demonstrated an antagonistic interaction between p53 and c-Myc involved in the regulation of M2 polarization. Specifically, p53 repressed the expression of Myc genes during M2 polarization. Given the well-established role of Myc in the control of anabolic metabolism, the results are consistent with the metabolic characteristics associated with M2 macrophages where glycolysis is downregulated whereas mitochondrial respiration is upregulated (Phan et al., 2017).

In tumorigenesis, tumor cells can substantially impact surrounding cells to shape the tumor microenvironment (TME) that promotes cancer progression. The dynamic interactions between tumor cells and immune cells have been widely reported. However, how p53 participates in regulating the tumor immune microenvironment is only beginning to be investigated. A recent study by Wang et al. showed that implanted mammary carcinoma cells acted on their surroundings in the host to induce an immunosuppressive microenvironment facilitating tumor growth (Wang et al., 2020). A contribution of p53 to the regulation of the immune microenvironment was demonstrated with a genetically engineered mouse model expressing a phospho-resistant MDMX. A prior study identified the 314-serine residue of MDMX as the phosphorylation site by receptor tyrosine kinases as well as the stress kinase p38. MDMX-S314 phosphorylation stabilized the MDM2/MDMX complex leading to augmented p53 degradation (de Polo et al., 2017). To investigate the effect of tumor cells on the p53 pathway in surrounding cells, the authors implanted an EO77 mammary carcinoma cell line that harbors mutant p53 into syngeneic host mice expressing wild-type p53. The implanted tumor cells imposed marked influence on the neighboring cells, evidenced by reduced p53 abundance in peritumor cells. This effect of the implanted tumor on peritumor cells appeared to be mediated by MDMX-S314 phosphorylation as the p53 decline in mice expressing *Mdmx*S314 A was blocked. Of significance were the observations that impediment of p53 decline was associated with mitigation of the suppression of immune responses as reflected by increased immune cell tumor infiltration and enhanced macrophage M1 polarization compared with that in wild-type mice.

Moreover, the improved immune response in *Mdmx*S314 A mice was coupled with a significant delay in tumor growth. Thus, the study implicates that tumor cells can induce an immune suppressive microenvironment by downregulating p53 in peritumor cells, suggesting a role of basal p53 in the maintenance of the immune response, However, further studies will be necessary to understand how basally expressed p53 preserves immune homeostasis.

Within the context of the tissue microenvironment, p53 was reported to play a role in maternal reproduction by controlling the expression of basal as well as inducible level of leukemia inhibitory factor (LIF), a cytokine critical for implantation (Hu et al., 2007).

P53-MEDIATED HOMEOSTATIC REGULATION OF CELL COMPETITION

Within tissues, cell-cell interactions are regulated by a host of mechanisms to preserve homeostasis. In addition to cell-intrinsic mechanisms to eliminate cells that contain unrepaired damages or are suboptimal, cells can also sense their neighbors to determine relative fitness, which constitutes an important mechanism to eliminate comparatively weaker cells, a process described as cell competition. Ample evidence indicates that cell competition is involved in various processes such as development, tissue homeostasis, and tumorigenesis. Cell competition to eliminate damaged and unhealthy cells are expected to yield positive and beneficial outcomes. It is, however, also conceivable that competition may contribute to tumor development. In accordance, studies have shown that malignant cells acquire various mutations to gain growth advantages in competition with neighboring normal cells (Vishwakarma and Piddini, 2020). While diverse mechanisms of cell competition have been reported, one of the widely observed pathways involves increased levels of Myc (Paglia et al., 2020). Myc is an important determinant of relative cell fitness, with winner cells having higher Myc levels than losers. However, despite these advances, the precise mechanism Myc affects cell fitness is not fully understood.

Given the homeostatic function of p53 and the well-established role of cell competition in preserving tissue homeostasis, it is probably not unexpected that p53 has been reported in the regulation of cell competition. Bondar et al. reported that a moderate increase in p53 induced by treatment with radiation at a low dose of 1Gy was associated with a loser phenotype in hematopoietic stem and progenitor cells (HSPCs) (Bondar and Medzhitov, 2010). The authors compared the ability of HSPCs with radiation-induced higher p53 levels versus non-irradiated controls to repopulate the chimeric bone marrow. The HSPCs with higher p53 levels were outcompeted by untreated HSPCs resulting in a marked reduction of p53 expressing HSPCs. In line with p53-mediated senescent function, high p53 expressing HSPCs were eliminated via the senescent program.

Like the modest p53 induction by treatment with low-dose radiation, a genetic method-induced mild increase in p53 also resulted in a less competitive status in both embryos and adult cells. Zhang et al. generated haploinsufficiency of Mdm2 and Mdm4 mice where p53 was slightly elevated but had little effect on growth (Zhang et al., 2017). However, mosaic haploinsufficiency of these genes rendered the cells with a competitive disadvantage during embryogenesis in mosaic embryos and adult tissues with active cell proliferation such as bone marrow, spleen, and testis. Of interest is the observation that the competitive disadvantage due to a mild increase in p53 levels was associated with reduced cell proliferation only in the developmental embryos but not in adult tissues, indicative of a context-dependent mechanism behind cell competition.

The finding that a moderate increase in p53 resulted in a less fit status would predict that reduced p53 level/activity might be associated with a more fit status. Indeed, in a study of embryonic development, knockdown p53 rendered embryonic stem (ES) cells a competitive advantage resulting in the replacement of wild-type ES cells when they were co-injected into the mouse embryo (Dejosez et al., 2013). While the study did not investigate how p53 downregulation could provide a competitive advantage, a recent study in mouse embryogenesis uncovered a novel mechanism of p53-mediated control of mTOR (Bowling et al., 2018). The authors demonstrated mTOR as a crucial determinant for cell competition during the early post-implantation stages. Higher mTOR activity provided a competitive advantage, whereas lower mTOR activity resulted in a disadvantage in competition. Of interest is the finding that p53 acted upon mTOR to control the activity of this metabolic enzyme. While elevated p53 repressed mTOR, reduced p53 expression by knockdown was associated with enhanced mTOR activity resulting in a marked increase in the competitive advantage. With the well-established metabolic function of mTOR, the study revealed a novel mTOR-dependent metabolic mechanism behind cell competition. Numerous studies have shown an antagonistic interaction between p53 and mTOR (Feng et al., 2005). For instance, p53 was reported to suppress mTOR activity by activating SESTRIN gene expression (Budanov and Karin, 2008) inducing the levels of REDD1 (Brugarolas et al., 2004). Further investigation is necessary to interrogate the functional interaction between basal p53 and the mTOR pathway.

P53-MEDIATED HOMEOSTATIC REGULATION OF STEM CELL SELF-RENEWAL AND DIFFERENTIATION

The p53-mediated homeostatic function also contributes to maintaining the balance between self-renewal and differentiation of stem cells (Jain et al., 2012). The early observation that in contrast with somatic cells, p53 is expressed at relatively high levels in mouse embryos or mouse embryonic stem cells (ESCs) (Schmid et al., 1991) suggests that p53 might function in early development and cell differentiation. However, subsequent studies revealed that the elevated p53 level in ESCs does not result in apoptosis or differentiation, primarily due to its cytoplasmic distribution. The subcellular p53 localization in ESCs was shown to be regulated by SIRT1-mediated deacetylation (Han et al., 2008) and might also be by MDM2/MDMX-mediated ubiquitination (Menéndez et al., 2011). The high level of p53 in the cytoplasm may keep it poised in response to potential stress. Indeed, DNA damage triggered by X-ray or UV irradiation induces p53 redistribution to the nucleus leading to p53 activation and subsequent induction of p53 target genes that promote ESCs differentiation (Lin et al., 2005). The available information supports the essential role of p53 in regulating the balance between pluripotency and differentiation in ESCs.

Similar to ESCs, p53 has been implicated in regulating induced pluripotent stem cells (iPSCs), which can be established by introducing reprogramming factors (OCT4, SOX2, KLF4, and c-MYC) into somatic cells. iPSCs display the ability of self-renewal and differentiation into many cell types, a feature like embryonic stem cells. Somatic cells undergo transitions in gene expression profile, epigenetic status, metabolic characteristics, and cellular morphology (Folmes et al., 2012). Ample evidence indicates that p53 functions as a barrier to somatic cell de-differentiation or reprogramming. Indeed, a recent study by Zhao et al. demonstrated that siRNA-mediated knockdown of p53 in human adult fibroblasts enhances iPS cell induction efficiency up to 100-fold (Zhao et al., 2008; Mathieu et al., 2014).

In line with the p53-mediated barrier function, the function of p53 in iPS is suppressed usually via a mode of post-translational modifications, which include ubiquitylation, acetylation, phosphorylation, methylation, or sumoylation of specific residues of p53 (Jain et al., 2012). Lee et al. reported that Aurora kinase A phosphorylates p53 (at Ser212 and Ser312) during iPS reprogramming, inhibiting p53 activity (Lee et al., 2012). Another study reported that Aurora kinase A-mediated p53 phosphorylation at Ser315 promoted MDM2-dependent ubiquitination and degradation of p53 protein (Katayama et al., 2004).

An additional type of modification frequently involved in the regulation of p53 in human ES cells is the acetylation of a lysine residue in the p53 protein. It was reported that despite being distributed in the nucleus of human ES cells, p53 is transcriptionally inactive because the 120/373 lysine residues are not acetylated. Although Sirt1 can maintain the non-acetylated status, a NAD-dependent deacetylase induced transcriptionally by Oct4 (Zhang et al., 2014), some lysine residues in the p53 protein can also be methylated, which often results in suppression of p53 transcription activity. Thus, it is conceivable that acetylation of certain lysine residues in the p53 protein is necessary for its transcription activity; methylation of the identical lysine residues would prevent their acetylation leading to p53 inactivation (Berger, 2010). Interestingly, preventing lysine methylation by replacing it with arginine at K370 R or K382 R resulted in p53 activation (Zhu et al., 2016), suggesting that p53 methylation-mediated p53 repression is not merely competing with activating acetylation.

Thus, it is clear that p53 activity is attenuated or inactivated in stem cells, which appears necessary to allow stem cells to replicate. The inactivation of p53 in stem cells can result from either a deficiency in p53 transcriptional activity or post-translational modifications on the p53 protein that result in an inactive p53 protein. Collectively, these studies suggest that p53 controls the transition between cell self-renewal and differentiation. p53 restricts the ability of somatic cells to undergo reprogramming into iPSCs.

The importance of metabolic regulation during the reprogramming to pluripotency has been well documented (Mathieu et al., 2014). Relative to their somatic counterparts, pluripotent stem cells, including ESCs and iPSCs, exhibit a high rate of glycolysis similar to aerobic glycolysis in cancer cells, which is necessary for maintaining stemness. This unique glycolytic metabolism in ESCs and iPSCs can provide bioenergetic supplies and promote the pentose phosphate pathway crucial for preserving redox homeostasis. Somatic cells undergo a metabolic switch from oxidative phosphorylation to glycolysis during reprogramming, which elicits the initiation and progression of reprogramming to iPSCs.

Ample evidence has shown that there is a very dynamic cross-talk between metabolic pathways and epigenetic programs. Cells continuously modify their metabolic programs and activities in response to nutrient availability, extracellular signals, and reprogramming/differentiation cues. Many intermediary metabolites can function as cofactors for epigenetic enzymes that catalyze histone methylation and acetylation reactions, contributing to the regulation of gene transcription. This cross-talk between intermediary metabolism and epigenetics has been demonstrated as central mechanisms by which metabolic pathways are engaged in stem cell fate determination (Kaelin and McKnight, 2013). Pluripotent stem cells are featured with bivalent chromatin regions, which encompass activating histone modifications, such as histone H3 lysine four trimethylations (H3K4me3), and repressive modifications histone H3 lysine 27 trimethylation (H3K27me3). Such bivalent chromatin domains enable developmental genes to maintain their repressive status without differentiation signals while allowing immediate activation in response to signal cues. Evidence indicates that epigenetic regulation of self-renewal and differentiation are intimately interfaced with cellular metabolism (Kaelin and McKnight, 2013). For instance, H3K4me3 is regulated by SAM levels generated through one-carbon metabolism (Shyh-Chang et al., 2013; Shiraki et al., 2014). Repressive H3K9me3 and H3K27me3 marks are regulated in an αKG-dependent manner through demethylation by JmjC-domain containing histone demethylases (JHDMs) and ten-eleven translocation (TET) enzymes (Kaelin and McKnight, 2013). With abundant evidence indicating an important role of p53 in regulating the balance between pluripotency and differentiation in stem cells, it will be interesting to link the p53 status to the metabolic regulation of stem cell fate. The correlation of reduced p53 nuclear abundance with glycolytic metabolism in stem cells is in line with the anti-glycolytic function of p53. Further studies will be necessary to understand better how a decrease in nuclear p53 abundance/activity can regulate metabolic pathways and the cross-talk with the epigenetic programs in stem cells.

THE P53-MEDIATED HOMEOSTATIC FUNCTION IN STRESS RESPONSE

A proper stress response is critical for maintaining homeostasis. When encountered with different levels of stress, cells have to determine the fate between survival and death. In response to excessive stress that is destructive to genome integrity and other cellular structures, cells must sense the intensity of damage and rapidly activate responses such as cell cycle arrest, DNA damage repair, senescence, or apoptosis if the damage is unrepairable. However, living cells or organisms are often exposed to

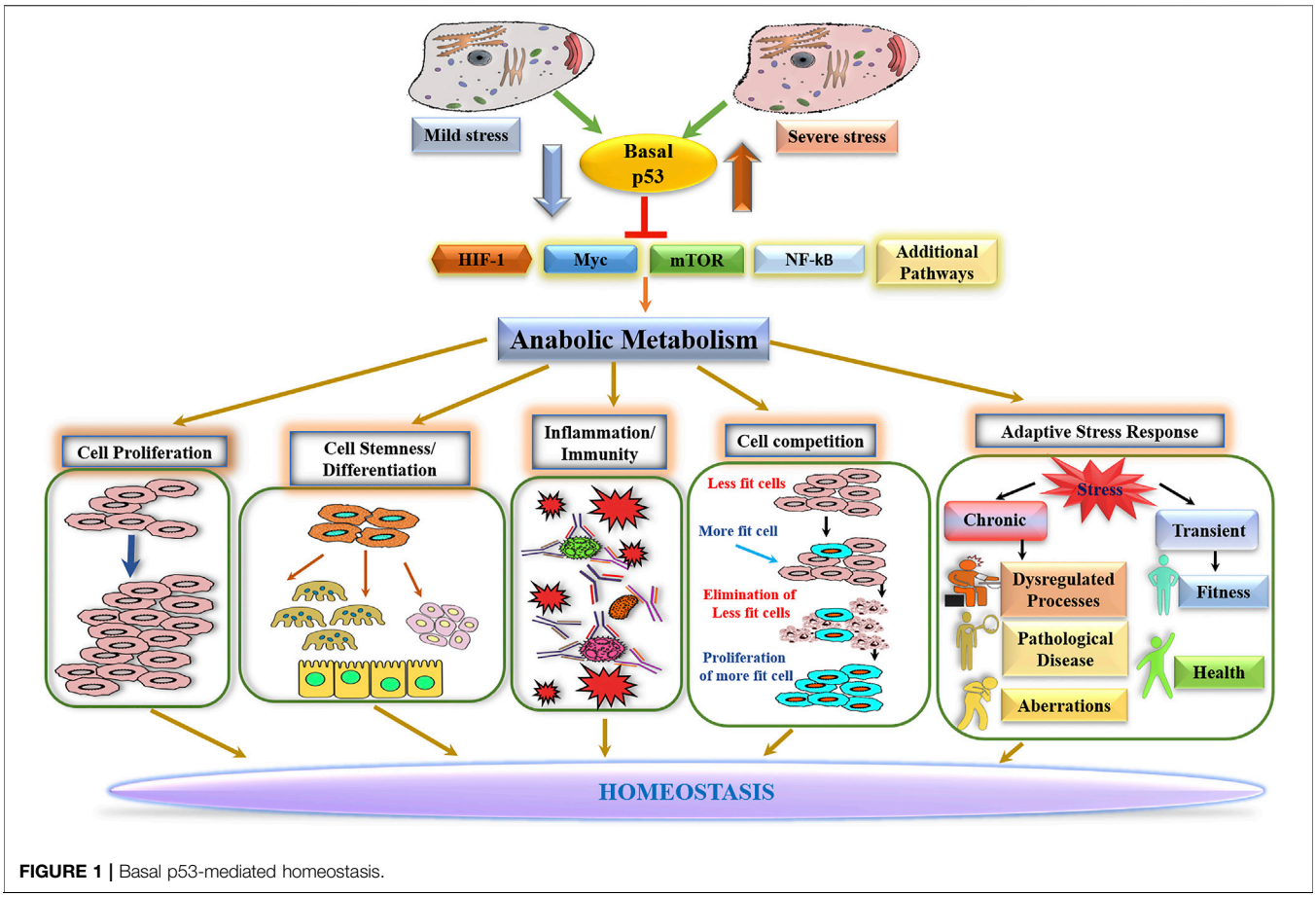

FIGURE 1 | Basal p53-mediated homeostasis.

temporary and low levels of stress in our daily lives. In response to such transient and mild stress, inciting cellular senescence or cell death would not make sense economically. Under such conditions, cells must finely tune their response to the perturbation based on stress level. Abundant evidence indicates that p53 is one of the key players in regulating the stress response (Kruiswijk et al., 2015). The importance of p53 in mediating cellular response to severe stress has been extensively investigated and relatively well understood. For instance, p53 is highly responsive to harsh conditions such as DNA damage, which activates p53 transcriptional activity, resulting in upregulation of genes whose products induce senescence or apoptosis to eliminate damaged cells. Relative to its contribution to defending organismal integrity under severe stress conditions, how p53 regulates responses to mild stress is not well studied and remains incompletely understood.

Available information indicates that mild stress can induce an adaptive response, an evolutionally conserved defense mechanism to preserve homeostasis (Calabrese et al., 2016). Evidence reveals that cellular adaptation to mild stress is an active process mediated by anabolic metabolism, which is critical in supporting cell viability and fueling the biosynthesis of biomolecules to mount the defense (Wang et al., 2019). While p53 was reported to be involved in the adaptive response (Horie et al., 2002; Lall et al., 2014), the underlying mechanisms are only beginning to be investigated. With the well-documented role of p53 in promoting oxidative phosphorylation while suppressing glycolysis, the anabolism-mediated adaptive response would suggest a compromised p53 activity. Indeed, it was reported that low-dose radiation-induced adaptive and protective response is associated with p53 downregulation (Lall et al., 2014), in line with p53's pro-death function. Of interest is that concurrent with low-dose radiation induced p53 downregulation is the upregulation of HIF1α and consequent induction of glycolysis and the pentose phosphate pathway. The study further showed that a low-dose radiation-induced metabolic switch is required for the protective adaptive response, consistent with an anabolism-dependent mechanism behind the adaptive stress response (Wang et al., 2019). Likewise, the low-dose arsenic-induced protective response is also associated with stimulation of metabolic reprogramming from oxidative phosphorylation to glycolysis, which is similarly mediated by p53 decline concurrent with however induction of NF-κB, which is known to induce the expression of several glycolytic genes (Ganapathy et al., 2014). The results suggest that basal p53 could keep anabolic metabolism in check and the downregulation of basal p53 becomes conducive for the induction of anabolic pathways. Given the critical contribution of HIF1α and NF-κB to the control of anabolic

metabolism, the antagonistic interaction between p53 and NF-κB (Ak and Levine, 2010), or p53 and HIF1α (Obacz et al., 2013) may represent an important mechanism for the metabolic regulation of the adaptive stress response, though the precise mechanisms by which basal p53 restrains HIF1α and NF-κB remain to be elucidated.

Studies have shown that the adaptive stress response is primarily mediated by a modest increase in ROS as treatment of cells with an antioxidant such as N-acetyl cysteine could mainly diminish the adaptive response (Ganapathy et al., 2014). The role of p53 in oxidative stress is well known. However, most studies have shown p53 activation by ROS (Kruiswijk et al., 2015). Evidence indicates that ROS-induced response depends on the level of ROS and the duration (Finkel 2012). Exposure to high levels of ROS and long durations can cause damage to DNA, RNA, or protein, whereas a transient increase of a modest amount of ROS would function as signal cues to induce a cellular response. It is conceivable that contrary to the high level of ROS that activates p53, a low level of ROS may stimulate signal pathways leading to p53 downregulation. ROS is known to primarily react with cysteine residues within target proteins, particularly low-pKa cysteine residues commonly found at the reactive site of enzymes (Finkel 2012). Protein phosphatases are well known extremely sensitive to be inactivated by ROS, resulting in activation of their target protein kinases (Finkel 2012). It was reported that protein kinases could downregulate p53 by phosphorylating MDMX increasing the MDM2/MDMX complex (Gerarduzzi et al., 2016; de Polo et al., 2017). Within the context of cellular metabolism, the association of anabolism-mediated adaptive stress response with p53 decline seems in line with p53-mediated repression of anabolic metabolism (Vousden and Ryan, 2009). Of note, the adaptive stress response can be beneficial when transient, however, persistent or chronic stress is usually associated with homeostatic imbalance, leading to pathological outcomes. While multiple factors might be involved, a sustained p53 downregulation during prolonged stress would likely contribute to the disruption of homeostasis and whereby development of diseases.

CONCLUSION

Homeostasis, a property crucial for normal physiology, is maintained by coordinated actions of diverse cellular processes and pathways. As a process fundamental to all biological functions, metabolism is intimately involved in regulating every facet of biological processes, which contributes to maintaining homeostasis. The basally expressed p53 safeguards homeostasis by keeping anabolic metabolism in check, which functions as a barrier to cell proliferation and governs numerous anabolism-dependent processes. In line with this notion are the observations that induction of many anabolism-driven processes is accompanied by a decline in nuclear p53 level/activity. While the ability of p53 to antagonize against Myc, HIF1α, NF-κB, or mTOR likely contributes to restraining anabolic metabolism (**Figure 1**), p53-mediated maintenance of metabolic homeostasis might involve a coordinated interaction of diverse processes at the systems level. Indeed, a recent study with genetically engineered mouse models revealed a high degree of connectivity between p53 and process-specific transcription factors (Mak et al., 2017). Of note is that most of the genes whose protein products are the key regulators and enzymes of metabolic pathways are extremely sensitive to changes in p53 protein levels, implicating that alterations in p53 abundance/activity may have very broad effects on metabolic programs. Further investigation is warranted to dissect the p53 network at the systems level to understand p53-mediated homeostatic function better.

AUTHOR CONTRIBUTIONS

Z-MY conceptualized the review, Z-MY and IN coordinated in drafting the manuscript.

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TP53 Mutation Infers a Poor Prognosis and Is Correlated to Immunocytes Infiltration in Breast Cancer

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Background: This study aimed to investigate the TP53 mutation, its potential immune features, its prognostic value, and its impact on immune infiltration in patients with breast cancer (BC).

Methods: We downloaded the somatic mutation data and clinicopathologic features of BC patients from the TCGA GDC database, UCSC Xena platform, and International Cancer Genome Consortium (ICGC) database. The association between the TP53 mutation, clinicopathology features, and overall survival (OS) in BC patients was analyzed. We evaluated the potential role of the TP53 mutation in the immune therapy response, including the tumor mutation burden (TMB), microsatellite instability (MSI), and tumor immune dysfunction and exclusion (TIDE). Moreover, ESTIMATE was employed to assess the ImmuneScore and StromalScore in BC patients. We also explored immunocyte infiltration related to the TP53 mutation and its potential mechanism. Immunohistochemistry (IHC) was performed to validate the association between the expression of CXCL1, CXCL10, and CCL20 and TP53 status.

Results: We found that the TP53 mutation was significantly associated with the shorter OS ($p = 0.038$) and was also an independent predictive factor of OS for BC patients ($p < 0.001$). Compared to that in the wild type group, the TP53-mutant group showed a higher TMB value ($P < 0.001$), MSI value ($p = 0.077$), and TIDE value ($p < 0.001$) with respect to BC patient immunotherapy. In addition, the ImmuneScore and StromalScore were both significantly increased in the TP53-mutant group (ImmuneScore: $p < 0.001$; StromalScore: $p = 0.003$). The results of CIBERSORT suggested that the TP53 mutation significantly promoted the infiltration of Tregs, T helper cells, and M0-type macrophages. KEGG and GSEA enrichment results suggested that the IL-17 signaling pathway and antigen processing and presentation pathways were significantly enriched in the TP53-mutant group. Importantly, based on IHC results of immune-related hub-genes, the chemokines CXCL1, CXCL10, and CCL20 were significantly upregulated in the TP53-mutant group in BC patients.

Conclusion: These results indicate that a TP53 mutation might serve as a biomarker for BC prognosis and is related to immunocyte infiltration in the tumor microenvironment.

Keywords: TP53, mutation, breast cancer, immunocytes infiltration, prognosis

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INTRODUCTION

Breast cancer (BC) is the most common malignancy in women and seriously threatens physical and mental health worldwide (Coughlin, 2019). It is currently estimated that there will be 276,480 newly diagnosed cases and 42,170 deaths from BC in the United States by 2020 (Le Blanc et al., 2020). According to histological characteristics, BC can be divided into HER2-positive, endocrine-dependent, and triple-negative breast cancer (TNBC) (Maughan et al., 2010). The treatment approaches should be based on the histological and molecular characteristics. Depending on the clinical subtype, therapeutic options include surgery, chemotherapy, endocrine therapy, and anti-HER2 targeting. However, 20–30% of BC cases still progress to distant metastases after diagnosis and treatment, and metastasis is the leading cause of death in approximately 90% of BC patients (Maughan et al., 2010; Britt et al., 2020). The tumor microenvironment (TME) is crucial for tumor progression and metastasis (Hinshaw and Shevde, 2019). The TME comprises not only cancer cells, but also the surrounding stromal cells and the tumor-infiltrating immune cells, and the immune cells play the leading role in the TME (Hinshaw and Shevde, 2019). With the development of immunotherapies with immune checkpoint blockade, the interaction between tumor and immune cells has come into focus (DeBerardinis, 2020). Recently, cancer treatment was revolutionized by immune checkpoint inhibitor (ICI) therapy owing to its durable clinical response, and ICI is usually considered in advanced metastatic BC (Santa-Maria and Nanda, 2018; Force et al., 2019). Nevertheless, some tumor tissues, especially TNBC, have a relatively low immune response after ICI treatment, which is mainly attributed to a "cold" immune microenvironment (Force et al., 2019). Thus, the exploration of new potential biomarkers to identify effective clinical therapy and improve the proportion of patients with BC responsive to ICI therapy must be solved.

The TP53 protein is a transcription factor that blocks tumor formation (Shahbandi et al., 2020). It is activated in response to several triggers, such as oncogene activation, DNA damage, hypoxia, and nutrient deprivation (Shahbandi et al., 2020). The TP53 protein serves as the guardian of the genome and monitors cell proliferation mainly by inducing DNA repair, cell-cycle arrest, and apoptosis (Baugh et al., 2018). Moreover, TP53 also contributes to other cellular processes, including angiogenesis, metabolism, stem cell maintenance, immune responses, and the cross talk between tumor cells and stromal cells TP53 (Baugh et al., 2018; Shahbandi et al., 2020). Nevertheless, the TP53 mutation is the most common mutation in BC, reported in 30% of BC and in 80% of TNBC cases (Silwal-Pandit et al., 2017). The TP53 mutation might alter the binding properties to its consensus sequence, and impair the transcriptional activation of TP53 target genes, which are involved in suppressing the tumor progression (Schon and Tischkowitz, 2018). Moreover, TP53-mutated tumors equip cells with novel tumor-promoting abilities, which include increased invasiveness, poor differentiation, and higher metastatic potential (Pitolli et al., 2019). Hancock et al. analyzed the molecular features of chemorefractory TNBC residual disease, and revealed that the TP53 mutations and MYC/TGFβ signaling pathway were the prominent drivers of recurrence, representing high-yield targets of the TP53 mutation (Hancock et al., 2019). These results suggest that TP53 mutation plays a vital prognostic role in BC.

Prior studies have indicated that TP53 status could shape the immune signatures by regulating the infiltration of the myeloid population, including neutrophils, macrophages, and monocytes (Blagih et al., 2020). Consequently, this upregulates the circulating neutrophils involved in tumor progression (Blagih et al., 2020). Further, cancer cells can modulate the TME through the secretion of cytokines and chemokines, and the TP53 mutation status drives the expression of CXCL1, CXCL10, and CCL20 (Addadi et al., 2010; Lowe et al., 2014). Other studies have suggested that significantly higher levels of immunocytes infiltrated into BC in patients with TP53 mutations compared to those with the wild-type phenotype, and TP53 mutation could promote the immunogenicity of tumors by regulating the TP53-related signaling pathways in BC (Li et al., 2019; Blagih et al., 2020). This might in part account for the mechanism through which TP53 mutations affect tumor immune infiltration. However, the significance of T53 mutations in BC therapy responses remains unclear. Presently, there is an urgent need to stratify patients according to TP53 status and evaluate the effects of TP53 mutations on predicting the efficacy of immunotherapy in BC.

In this study, we downloaded the somatic mutation data of BC from the TCGA GDC database and evaluated the relationship between the tumor mutation burden (TMB) and TP53 status in BC. Moreover, BC patients were divided into "TP53-mutant" and "TP53-wild-type" groups, to explore the differentially expressed genes (DEGs) related to TP53 mutations. Then, the functional enrichment analysis and gene-set enrichment analysis (GSEA) were performed to reveal the signaling pathways and biological processes associated with DEGs in TP53-mutant BC. We also constructed protein-protein interaction and mRNA-miRNA-lncRNA ceRNA network for hub-genes using Cytoscape and miRTarBase, respectively. Importantly, we also validated the association between hub-genes expression which related to TME and TP53 status by immunohistochemistry (IHC) in cancer tissues of BC patients. Further, we quantified the immune cells proportions in the TCGA-BRCA samples and compared the differences in the immune cell infiltration in tumor tissues between TP53-mutant and TP53-wild type (TP53-wt) groups. Additionally, we conducted Cox regression analysis to identify the prognostic role of the TP53 status with BC progression, and constructed a nomogram including TP53 status to predict the overall survival (OS) of BC patients.

MATERIALS AND METHODS

Data Downloading and Bioinformatic Analyses

We obtained somatic mutation data of breast invasive carcinoma (BRCA) samples from the TCGA GDC database by choosing the "Masked Somatic Mutation" (https://portal.gdc.cancer.gov/)

(Zhang et al., 2021). The preprocessing was employed with VarScan software and the somatic mutations were visualized using the MAFtools R package (Mayakonda et al., 2018). Then, we downloaded the RNA sequencing data (FPKM values) of the BC patients and subsequently converted FPKM values to TPM values. Moreover, the data were divided into the lncRNA and mRNA expression profiles. Further, we download the clinicopathologic features and outcomes in the same population from the UCSC Xena platform (http://xena.ucsc.edu/), such as sex, age, stage, and microsatellite instability (MSI) status (Speir et al., 2016). In addition, two datasets including somatic mutation and clinical data in BC patients were downloaded from the International Cancer Genome Consortium (ICGC) database (https://daco.icgc.org/), which were Breast Cancer-FR (BRCA-FR) and Breast Cancer-KR (BRCA-KR) (Zhang et al., 2019).

Copy Number Alteration Analysis

To analyze the copy number variations (CNVs) of TP53 in TCGA-BRCA patients, we obtained the data of Masked Copy Number Segment using the TCGAbiolinks package in R language (Colaprico et al., 2016). The CNV data was processed using GISTIC 2.0 by performing the GenePattern5 function (Reich et al., 2006). During the analytical process, GISTIC 2.0 with default settings was used except for several parameter (i.e., the confidence was 0.99 and X chromosome was included before the analysis). Finally, the results of GISTIC 2.0 were visualized with the MAFtools R package.

Correlations Between Somatic Mutation and Tumor Mutation Burden

To predict the response to ICI therapy caused by the TP53 mutation in BC patients, we computed the TMB, MSI, and tumor immune dysfunction and exclusion (TIDE, http://tide.dfci.harvard.edu) for each BC sample. The total number of the somatic mutations per megabase of the genome detected in the tumor was defined as the TMB (Yarchoan et al., 2017); the insertion or deletion of repeat units results in a change in the microsatellite length, which is referred to as MSI (Vilar and Gruber, 2010); TIDE is a computational framework that can evaluate the response to immunotherapy and predict tumor immune escape by analyzing the gene expression profiles of cancer cases (Jiang et al., 2018). We calculated all TMB, MSI, and TIDE values for each sample, and compared their differences between patients with wild-type TP53 and those with mutant TP53 using a Wilcoxon rank-sum test.

Relationship Between Clinical Features and Differentially Expressed Genes

To explore the significance of mutant TP53 in BC progression, we classified the TCGA patients into "TP53-mutant" and "TP53-wt" groups. The holistic analysis was employed by principal component analysis (PCA), which is a multivariate statistical technique under the broad title of factor analysis, that focus on pattern recognition and signal processing (Ringnér, 2008). PCA was conducted with the R packages factoextra and FactoMineR. DEGs were determined using the Bioconductor R package DESeq2 (Love et al., 2014), and the threshold for DEGs was $p < 0.01$ and $|\log FC| > 1.5$. The results were presented in heatmap and volcano plots.

Functional Enrichment Analysis and Gene-Set Enrichment Analysis

Gene ontology (GO) analysis is a common bioinformatics tool applied in large-scale functional enrichment studies that can annotate genes and analyze the biological process, cellular component, and molecular function of these genes (Yu et al., 2012). Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) is a database to explore the comprehensive biological systems and functions generated by experimental techniques in high-throughput biology from massive molecular datasets (Yu et al., 2012). The GO annotation and KEGG pathway enrichment analyses of signature genes was implemented using the ClusterProfiler package and the DAVID online database (Yu et al., 2012). Results with a false discovery rate (FDR) less than 0.05 were considered statistically significant.

To investigate the differences in biological processes between TP53-mutant and TP53-wt groups, we performed GSEA, based on the gene expression profile of the TCGA-BRCA dataset. GSEA is also a functional enrichment analysis, based on a predefined set of genes between two groups, which can determine whether there is a statistical difference (Subramanian et al., 2005). It is used frequently in analyzing the enrichment of signaling pathways and biological processes. The geneset of c2. cp.kegg.v6.2.-symbols was downloaded from the Molecular Signature Database (MsigDB, http://software.broadinstitute.org/gsea/msigdb/). GSEA was performed, and adjusted *p*-values less than 0.05 were regarded as statistically significant.

Comparison of Immune Cell Infiltration and Immune Scores Between Two Groups

To quantify the immune cell proportions in the TCGA-BRCA samples, we used the CIBERSORT algorithm (https://cibersort.stanford.edu/) and the LM22 gene signature matrix (Newman et al., 2015). Highly sensitive and specific discrimination was performed for the phenotypes of 22 immunocytes (T cells, B cells, natural killer cells, and macrophages) in the TME (Hinshaw and Shevde, 2019). CIBERSORT was run to deconvolute samples, and used the expression values of a set of reference genes (547 genes), which were considered the minimal representative values for each type of cells. Based on these values, we deduced the cell type proportioning from the data of samples with mixed cells. Thus, we analyzed the effect of TP53 gene mutations on immune cell infiltration in TCGA-BRCA patients.

Meanwhile, the ESTIMATE algorithm was applied to assess the immune infiltration levels of BC patients according to the interpretation of gene expression profiles (Yoshihara et al., 2013). The ImmuneScore and StromalScore were calculated for each sample using the using the ESTIMATE package in R (https://www.r-project.org/). We performed Mann-Whitney U tests to

compare the differences in immune cell infiltration in tumor tissues between TP53-mutant and TP53-wt groups.

Construction of Protein-Protein Interaction Network and Identification of Hub-Genes

In this study, we implemented the STRING (https://string-db.org) (Szklarczyk et al., 2019) to infer the protein-protein interaction (PPI) network. STRING is an online tool that can predict protein-protein interactions and construct the PPI network of selected genes. Interactions with a confidence score greater than 0.7 were included to construct the PPI network in Cytoscape software (Version 3.7.2). We defined the high-density areas as hub-genes based on the vertex-weighting scheme by using the MCODE plugin (Shannon et al., 2003).

Construction of mRNA-miRNA-lncRNA ceRNA Network

The miRNA-mRNA interaction data was downloaded from the mirTarBase (http://mirtarbase.mbc.nctu.edu.tw/index.php) (Hsu et al., 2011). Then, we predicted the target miRNAs of the hub-genes from the PPI network, and carried out using the miRTarBase (http://mirtarbase.mbc.nctu.edu.tw) (Hsu et al., 2011). Moreover, the regulatory relationships between miRNA and lncRNA were further established. Based on these hub gene-miRNA pairs and miRNA-lncRNA pairs, a ceRNA network for mRNA-miRNA-lncRNA was illustrated using Cytoscape software (version 3.7.2) (Shannon et al., 2003).

Immunohistochemistry

To validate the association between the expression of CXCL1, CXCL10, and CCL20 and TP53 status, we collected 10 cancer tissues with TP53 mutation and 10 tissues without mutation from BC patients. We performed IHC to compare the level of CXCL1, CXCL10, and CCL20 between two groups. IHC was performed as previously described (Wang et al., 2021), with antibodies specific for TP53 (Affinity, 1:100), CXCL1 (Affinity, 1:100), CXCL10 (Affinity, 1:100), or CCL20 (Affinity, 1:100). Pictures were taken with a microscope (Nikon DS-Ri2, Tokyo, Japan). Pathological samples were evaluated and scored separately by two qualified pathologists. The IHC scoring is as follows: 0 for no staining, 1+, 2+, 3+, and 4 + for 1–24, 25–49%, 50–74%, and over 75% staining intensity, respectively.

Analysis of Anti-Cancer Drugs Sensitivity

Genomics of Drugs Sensitivity in Cancer (GDSC) is a public online database (http://www.cancerrxgene.org/downloads/) and is used to determine anticancer drug response and somatic mutations in cancer (Yang et al., 2013). We identified the association between TP53 mutations and anticancer drug sensitivity in BC patients, based on the data of the gene mutation status in cancer cell lines and IC50 values of anticancer drugs.

Construction of TP53-Mutation Prognostic Model

To identify the prognostic role of TP53 status based on clinicopathological features, we analyzed the OS rate by conducting univariate and multivariate Cox regression analyses to test the risk score. The potential prognostic parameters were included to construct a nomogram using the TCGA-BRCA datasets. We constructed the nomogram using the rms R package. To analyze the performance of models, a calibration plot was graphically mapped by the nomogram predicted vs. observed probability. Moreover, the concordance index (C-index) was commonly obtained to quantitatively examine the discrimination ability of the nomogram.

Statistical Analysis

In this study, all data processing and analysis were carried out using R software (Version 4.0.2). For continuous variables, a Student's t-test was used to compare the means between the normally distributed variables, whereas a Mann-Whitney test was used for the variables that were not normally distributed. Moreover, a Chi-square test or Fisher exact test was used for discontinuous variables. The correlations among genes were determined by Pearson correlation analysis. Prognostic analysis was performed using the R package survival. The Kaplan-Meier curves were plotted to show the survival time of BC patients, and the log-rank test was used for the survival comparisons between the two groups. The independent prognostic factors in BC were identified using univariate Cox regression and multivariate Cox regression analyses. We plotted the receiver operating characteristic (ROC) curves using the pROC R package (Robin et al., 2011). The area under the ROC curve (AUC) was calculated to assess the prognostic risk scores (Robin et al., 2011). A two-sided p value less than 0.05 was considered as statistically significant.

RESULTS

Overall Mutation Analyses of Breast Cancer Patients

To analyze the effects of TP53 mutations on the genomic mutations in BC patients, we downloaded three BRCA datasets from TCGA and ICGC databases (n = 943). First, we evaluated the mutation profile in BRCA patients as shown in **Figure 1A**. The results indicated that the missense mutations accounted for a major portion, single nucleotide polymorphisms (SNPs) were more often observed than insertion-deletion (indel) mutations, and the C > T single nucleotide variants were the most common variant in BC patients. The frequency of TP53 mutations was the second most in all the BRCA patients. Subsequently, we subdivided all patients into two groups, TP53-mutant and TP53-wt groups, according to the TP53 status. The somatic mutations of BRCA samples were calculated and visualized by the "Maftools" R package, and were presented in **Figure 1B**. The waterfall plots presented the mutation profile of associated genes (**Figure 1B** for TCGA-BRCA; **Figure 1C** for BRCA-FR; and **Figure 1D** for BRCA-KR). Moreover, the amino acid substitutions in the TP53 gene were evaluated and shown in **Figure 1E**. The location of each amino acid variant was corresponding to the coordinate axis below. The mutation type was distinguished by different colors, and the tag indicates the meaning of each color. The results

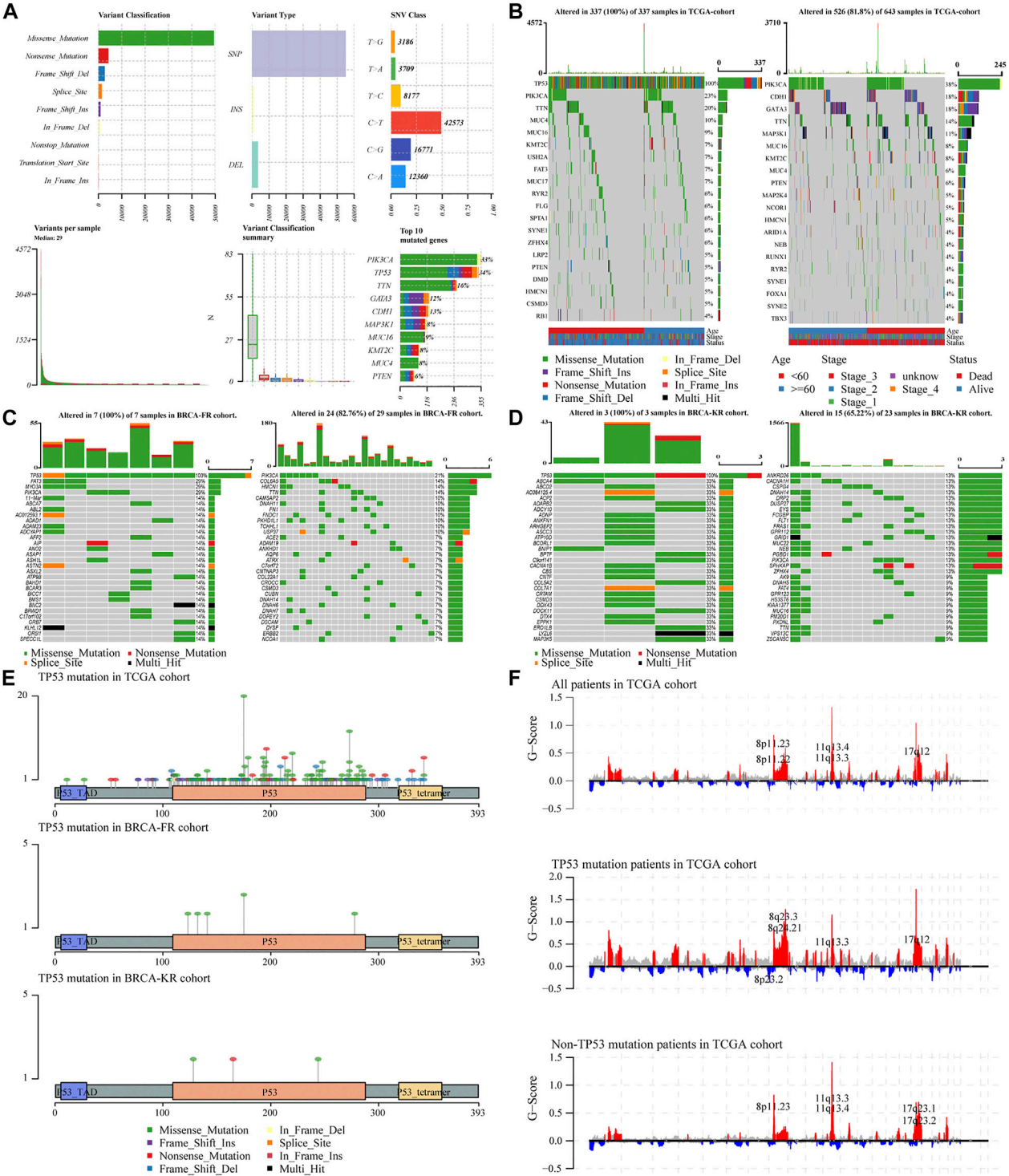

FIGURE 1 | Characteristic of TP53 mutation in TCGA-BRAC patients. Overall information of somatic mutation of BRCA patients **(A)**; the top 30 significant mutations were found in the TCGA-BRCA cohort, of which the left side was TP53-mutant group and the right side was TP53-wt group **(B)**; the distribution of the top 30 significant mutations in the dataset of BRCA-FR **(C)** and BRCA- KR **(D)**; the distribution of amino acids in TP53 protein in TCGA-BRCA, BRCA-FR, and BRCA-KR data sets **(E)**; the results of CNV for TCGA-BRCA were visualized by MAFtools **(F)**. SNP, single nucleotide polymorphism; SNV, single nucleotide variant; CNV, copy number variation.

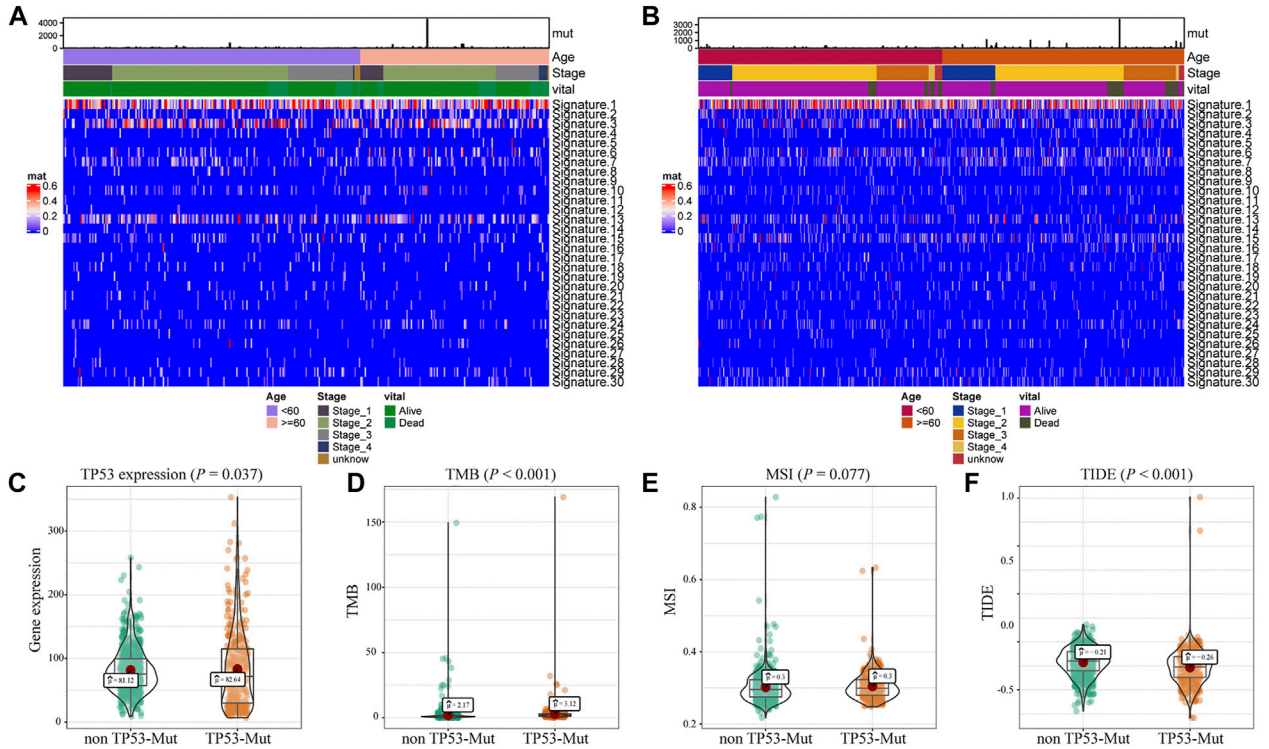

FIGURE 2 | Analysis of biological characteristics of TP53 mutation in BC patients. The cluster analysis of cosmic signature in patients with TP53 mutation and the clinical features of patients were shown by the heatmap **(A)**; the cluster analysis of cosmic signature in patients without TP53 mutation in TCGA-BRCA dataset **(B)**; compared with TP53-wt group, the expression level of TP53 was significantly increased in TP53 mutation group of the BC patients **(C)**; the TMB level of TP53-mutation patients was significantly increased **(D)**; the MSI value of TP53 mutation patients was significantly increased **(E)**; TIDE in patients with TP53 mutation was significantly higher **(F)**. TMB, tumor mutation burden; MSI, microsatellite instability; TIDE, tumor immune dysfunction and exclusion.

showed that the main mutant form of TP53 amino acid was missense mutation in all three datasets. We also separated TCGA-BRCA patients into TP53-mutant and TP53-wt groupsTP53, and analyzed the CNV status. The data were analyzed via GISTIC 2.0 to obtain gene-level estimates of CNV, with the default settings except for several parameters (e.g., confidence: 0.99; X chromosome was not excluded from the analysis). Finally, the GISTIC 2.0 output was visualized using the MAFtools package, and shown in **Figure 1F**. This indicated that significant alterations in CNV levels located in related genes were observed in the TP53-mutant group.

Association Between TP53 Mutation and Immunotherapy Indicators

Further, we explored the biological effect of TP53 mutations based on the mutational signature analysis. According to the biological characteristics, somatic mutational processes could be characterized by the mutation patterns, and 96 mutation patterns were translated into 30 different mutational signatures (Alexandrov et al., 2020). The results indicated that significant changes in Signature 1, 3, and 13 were observed compared to those in the TP53-wt group (**Figures 2A,B**). In addition, compared with that in the TP53-wt group, the level of the TP53 gene was substantially increased in the TP53-mutant group ($p = 0.037$; **Figure 2C**), and the TMB value ($p < 0.001$; **Figure 2D**), MSI value ($p = 0.077$; **Figure 2D**), and TIDE score for immunotherapy ($p < 0.001$; **Figure 2E**) were also elevated in the TP53-mutant group.

Analysis of Drug Sensitivity in Breast Cancer Patients with the TP53 Mutation

To detect the effect of TP53 mutations on drug sensitivity in BC patients, we assessed the correlation between TP53 mutations and IC50 values of molecules from the GDSC database. The result showed that multiple drugs related to the frequency of TP53 mutation (**Figure 3A**). The pathway analysis revealed that the TP53 pathway was significantly enriched (**Figure 3B**), and the high mutation rates of 6 genes in this pathway were also prevalent in BC patients (**Figure 3C**). Moreover, the TP53 mutation had some effect on BC sensitivity to multiple chemotherapy agents and small molecule substances (**Figure 3D**), especially to Nutlin-3a (**Figure 3E**).

Differential Gene Expression Analysis in Breast Cancer Patients

To assess the effect of the TP53 mutation on BC tumorigenesis, the TCAG-BRCA patients were separated into TP53-mutant and TP53-wt groups. As shown in **Table 1**, TP53 mutation status was significantly correlated with a younger age (<60 vs. ≥ 60,

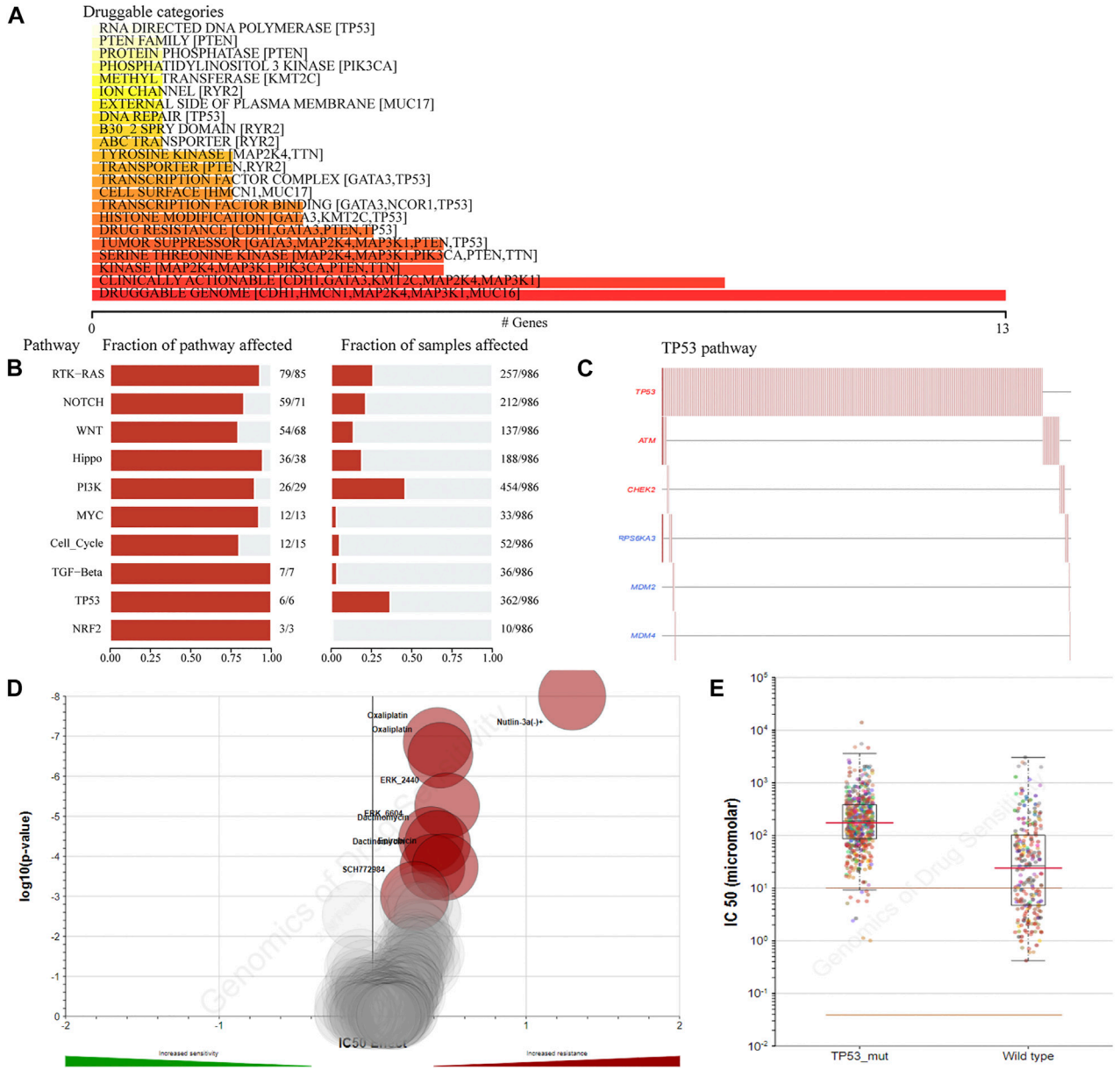

FIGURE 3 | The drug sensitivity caused by TP53 mutation. The relationship between gene mutation level and different kinds of drugs in TCGA-BRCA dataset was analyzed **(A)**; analysis of gene mutation level in different carcinogenic signaling pathways in TCGA-BRCA dataset **(B)**; mutation distribution of six major genes in TP53 signaling pathway in TCGA-BRCA patients **(C)**; the volcano map shows the sensitivity analysis of TP53 gene mutation to different anticancer chemotherapy drugs; the red circle indicates that TP53 gene mutation leads to the decrease of drug sensitivity **(D)**; compared with TP53-wt group, the IC50 value of nutlin-3a (-) in patients with TP53 mutation was significantly higher **(E)**. wt, wild type.

$p = 0.007$) and earlier M stage (M0 vs. MX, $p = 0.007$). As evaluated by PCA analysis, significant differences were shown ($p < 0.05$) between TP53-mutant and TP53-wt groups (**Figure 4A**). Moreover, DEGs analysis identified that 845 upregulated DEGs and 237 downregulated DEGs were associated with the TP53 mutation (|log2 fold change|> 1.5 and (adjust) p-value < 0.01; **Figures 4B,C**).

Subsequently, to analyze the cellular functions of 1082 DEGs, we conducted GO and KEGG enrichment analyses using the R package clusterProfiler. The results of the GO analysis demonstrated that DEGs were involved in the biological processes of cornification, keratinization, skin development, intermediate filament cytoskeleton, and peptidase inhibitor activity (**Table 2**; **Figures 4D–F**). KEGG pathway analysis suggested that the immune-related DEGs were significantly enriched in neuroactive ligand-receptor interaction, nicotine addiction, salivary secretion, and the IL-17 signaling pathway (**Table 3**; **Figure 4G**). Besides, the GSEA results of the TP53-mutant group revealed that the significant pathways ($p < 0.05$ and FDR q-value < 0.25) were enriched in

TABLE 1 | Association between TP53 status and clinical pathologic features in TCGA-BRCA patients.

Variables	All patients (n = 943)	TP53-wt (n = 624)	TP53-mutant (n = 319)	*p* value
Age	—	—	—	0.007
< 60	517 (54.8%)	322 (51.6%)	195 (61.1%)	—
≥60	426 (45.2%)	302 (48.4%)	124 (38.9%)	—
Pathologic stage	—	—	—	0.961
I and II	713 (75.6%)	471 (75.5%)	242 (75.9%)	—
III and IV and X	230 (24.4%)	153 (24.5%)	77 (24.1%)	—
T	—	—	—	0.719
T1 and T2	803 (85.2%)	529 (84.8%)	274 (85.9%)	—
T3 and T4 and TX	140 (14.8%)	95 (15.2%)	45 (14.1%)	—
N	—	—	—	0.601
N0 and N1	767 (81.3%)	511 (81.9%)	256 (80.3%)	—
N2 and N3 and NX	176 (18.7%)	113 (18.1%)	63 (19.7%)	—
M	—	—	—	0.042
M0	788 (83.6%)	510 (81.7%)	278 (87.1%)	—
M1 and MX	155 (16.4%)	114 (18.3%)	41 (12.9%)	—

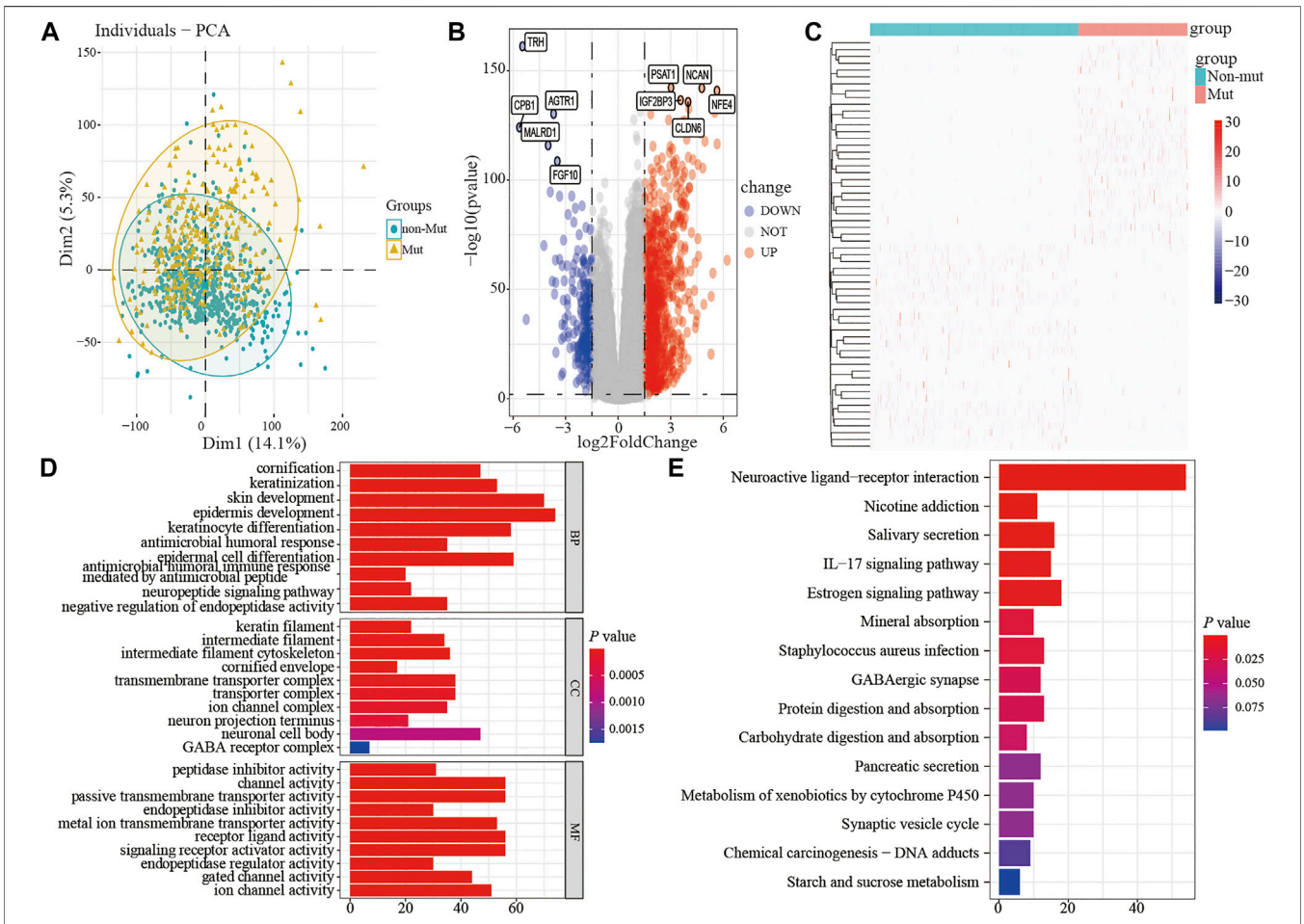

FIGURE 4 | The differential expression and functional enrichment analysis based on TP53 mutation. PCA was performed between the patients with mutation and non-mutation **(A)**; volcano map and heatmap showed the expression of DEGs between TP53-mutation and TP53-wt group **(B-C)**; the results of GO analysis included the cell component **(D)**, biological process **(E)**, and molecular function **(F)** terms; the results of KEGG analysis that differentially expressed genes were involved in biological related signaling pathways **(G)**. PCA, principal component analysis; wt, wild type.

TABLE 2 | Top three clusters with their representative enriched terms of GO analysis.

GO	ID	Description	Count	*p* value	Gene
BP	GO:0070268	Cornification	47	4.11E-32	KRT16/KRT83/PI3/DSG1/DSC2/KRT9/KRT6B/KRT79/TGM1/KRT86/KRT6A/KLK5/DSG3/KRT6C/CASP14/KLK14/SPINK5/DSC3/KRT81/PKP1/TGM5/KRT37/KRT4/SPINK6/KRT34/KRT23/KRT78/KRT5/KRT31/KRT1/KRT3/KRT17/IVL/KRT14/KRT75/KRT35/KRT84/LIPK/KRT85/KRT77/KRT82/SPRR1B/KRT33B/SPRR2G/SPRR2D/SPRR2E/LCE3D
BP	GO:0031424	Keratinization	53	3.39E-22	KRT16/KRT83/PI3/DSG1/DSC2/KRT9/KRT6B/KRT79/TGM1/KRT86/CDH3/KRT6A/KLK5/DSG3/KRT6C/CASP14/KLK14/SPINK5/DSC3/KRT81/PKP1/TGM5/KRT37/KRT4/SPINK6/KRT34/KRT23/KRT78/KRT5/KRT31/KRT1/KRT3/KRT17/IVL/KRT14/KRT75/KRT35/KRT84/KRTAP3-3/LIPK/KRT85/KRT77/KRT82/KRTAP1-1/LCE3A/SPRR1B/KRT33B/SPRR2G/SPRR2D/SPRR2E/LCE3D/SPRR4/KRTAP4-1
BP	GO:0043588	Skin development	70	1.12E-19	FOXC1/FGF10/KRT16/KRT83/PI3/DSG1/DSC2/EGFR/CTSV/KRT9/KRT6B/GAL/KRT79/TGM1/KRT86/CDH3/KRT6A/KLK5/FERMT1/CLDN1/SCEL/GJB3/DSG3/KRT6C/FOXQ1/CASP14/LHX2/KLK14/SOSTDC1/SPINK5/LGR5/DSC3/EDAR/KRT81/DKK1/PKP1/TGM5/KRT37/KRT4/SPINK6/KRT34/KRT23/KRT78/KRT5/KRT31/KRT1/KRT3/KRT17/IVL/KRT14/KRT75/KRT35/KRT84/KRTAP3-3/LIPK/KRT85/KRT77/KRT82/S100A7/KRTAP1-1/LCE3A/SPRR1B/KRT33B/SPRR2G/SPRR2D/SERPINB13/SPRR2E/LCE3D/SPRR4/KRTAP4-1
CC	GO:0045095	Keratin filament	22	8.00E-10	KRT83/KRT6B/KRT79/KRT86/KRT6A/KRT6C/CASP14/KRT81/KRT4/KRT78/KRT5/KRT1/KRT3/KRT14/KRT75/KRT84/KRTAP3-3/KRT85/KRT77/KRT82/KRTAP1-1/KRTAP4-1
CC	GO:0005882	intermediate filament	34	1.23E-09	INA/KRT16/KRT83/KRT9/KRT6B/KRT79/KRT86/KRT6A/KRT6C/KRT222/CASP14/KRT81/PKP1/KRT37/KRT4/KRT34/KRT23/KRT78/KRT5/KRT31/KRT1/KRT3/KRT17/KRT14/KRT75/KRT35/KRT84/KRTAP3-3/KRT85/KRT77/KRT82/KRTAP1-1/KRT33B/KRTAP4-1
CC	GO:0045111	Intermediate filament cytoskeleton	36	6.84E-09	INA/KRT16/KRT83/KRT9/KRT6B/KRT79/SLC1A6/KRT86/KRT6A/S100A8/KRT6C/KRT222/CASP14/KRT81/PKP1/KRT37/KRT4/KRT34/KRT23/KRT78/KRT5/KRT31/KRT1/KRT3/KRT17/KRT14/KRT75/KRT35/KRT84/KRTAP3-3/KRT85/KRT77/KRT82/KRTAP1-1/KRT33B/KRTAP4-1
MF	GO:0030414	Peptidase inhibitor activity	31	3.30E-09	A2ML1/RARRES1/PI3/SLPI/NLRP7/CST9L/SERPINB7/CST5/SERPINB5/SPINK5/CST2/CARD17/UMODL1/HMSD/CST9/SPINK6/SERPINB2/SERPINB4/SERPINA11/SERPINB12/MT3/CST4/CARD18/FETUB/SMR3B/SERPINA6/SERPINB3/OPRPN/SMR3A/SERPINB13/CSN2
MF	GO:0015267	Channel activity	56	4.50E-09	SLC26A9/TRPM8/TTYH1/TMC3/GRIA1/KCNS1/KCNQ4/KCNK5/KCNG1/CLCN4/KCNB2/CHRNA9/GABRP/GRIA2/KCNE4/GABRA5/CHRNA5/HTR3A/GABRE/CNGB1/GJB3/KCNE5/KCNK9/CNGA1/GRIN2B/CACNA1B/TRPV3/GLRA3/SCN7A/TRPV6/CNGA3/KCNH1/GJB7/KCNC1/AQP5/ABCC8/KCNJ4/CLIC6/KCNC2/GABRG3/GABRQ/KCNV1/KCNF1/UNC80/GJB4/CLCA2/ASIC2/OTOP1/KCNJ3/CACNG5/GABRA3/KCNJ18/KCNK16/AQP12B/HTR3B/CLCA1
MF	GO:0022803	Passive transmembrane transporter activity	56	4.86E-09	SLC26A9/TRPM8/TTYH1/TMC3/GRIA1/KCNS1/KCNQ4/KCNK5/KCNG1/CLCN4/KCNB2/CHRNA9/GABRP/GRIA2/KCNE4/GABRA5/CHRNA5/HTR3A/GABRE/CNGB1/GJB3/KCNE5/KCNK9/CNGA1/GRIN2B/CACNA1B/TRPV3/GLRA3/SCN7A/TRPV6/CNGA3/KCNH1/GJB7/KCNC1/AQP5/ABCC8/KCNJ4/CLIC6/KCNC2/GABRG3/GABRQ/KCNV1/KCNF1/UNC80/GJB4/CLCA2/ASIC2/OTOP1/KCNJ3/CACNG5/GABRA3/KCNJ18/KCNK16/AQP12B/HTR3B/CLCA1

focal adhesion, ribosome, antigen processing and presentation, and ECM receptor interaction, and the details are shown in **Table 4** and **Supplementary Figure S1A, S1B**.

Protein-Protein Interaction and ceRNA Network

The PPI network of DEGs was constructed using the STRING online database (**Supplementary Figure S2A**), and the results were imported into Cytoscape software for further analysis (**Supplementary Figure S2B**); the red color represented up-regulated gene expression and the blue color represented down-regulated gene expression. Then, we used the plugin MCODE in Cytoscape to analyze the important modules. In the regions of high density, the central nodes were identified as hub-genes (**Supplementary Figure S2C**). Based on the information of miRNA-mRNA interactions in the miRTarBase, we predicted the miRNAs associated with the hub-genes, and lncRNA associated with the miRNAs. Thus, the mRNA-miRNA-lncRNA ceRNA network was constructed based on the predicted relationship shown in **Supplementary Figure S2D**. The results above indicated that the chemokines CXCL1, CXCL10, and CCL20 was significantly upregulated in the TP53-mutant group (**Supplementary Figure S2C, S2D**). Further, the CXCL10 and CCL20 expression level was lower in BC tissues (TCGA-BRCA patients) compared with normal tissues (**Figures 5A,B**). We also examined the expression level of them in paired tissue samples. The results indicated that the level of CXCL10 and CCL20 in BC tissues was also significantly lower than those in paired samples (**Figures 5C,D**).

TABLE 3 | Top nine clusters with their representative enriched terms of KEGG analysis.

ID	Description	Count	*p* value	Gene
hsa04080	Neuroactive ligand-receptor interaction	53	1.35E-15	7,200/185/887/165,829/1131/9,568/2890/4,986/51,083/6019/55,584/2568/2,891/4886/2,558/1138/5,697/2564/1394/64,106/5746/5,646/3362/2,904/8001/4,923/2692/4,887/10,874/7434/2,567/55,879/2691/4,889/553/6863/797/4922/1081/57,152/885/5540/4,543/117,579/2556/7,201/5173/796/84,539/9248/3,358/1443/2,689
hsa05033	Nicotine addiction	11	1.12E-06	2,890/2568/2,891/2558/2,564/2904/774/2567/55,879/2556/57,084
hsa04970	Salivary secretion	16	4.07E-06	1131/4025/1473/477/1470/492/55,503/480/362/653,247/1755/3346/1472/51,806/277/5,542
hsa04657	IL-17 signaling pathway	15	2.09E-05	6,374/2919/6,279/3627/6,364/3934/6,354/6280/3,576/5596/4,312/6372/338,324/6278/1673
hsa04915	Estrogen signaling pathway	18	5.38E-05	3,868/9568/1956/3857/399,694/2099/8688/3,885/25,984/7031/5,241/3881/3,872/3861/3,886/3760/51,806/3,884
hsa05150	*Staphylococcus aureus* infection	13	0.000403	3,868/1828/3857/1672/8688/3,885/25,984/3881/3,872/3861/3,886/3884/1673
hsa04727	GABAergic synapse	12	0.000695	9,568/2568/18/2558/2,564/774/10,991/2571/2,567/6538/55,879/2,556
hsa04974	Protein digestion and absorption	13	0.000799	1302/1360/1299/59,272/6564/1297/8645/169,044/136,227/5646/477/480/256,076
hsa04973	Carbohydrate digestion and absorption	8	0.001174	93,432/3938/8,972/477/480/80,201/57,818/277

Relationship Between Hub-Genes Expression and TP53 Status

We determined the effects of TP53 mutation on the expression of hub-genes by IHC in BC tissues, including CXCL1, CXCL10, and CCL20. As shown in **Figure 5E**, the upregulated expression of CXCL1, was significantly associated with TP53 mutation ($p < 0.05$). The similar results were also found for the expression of CXCL10, CCL20, and TP53 ($p < 0.05$; **Figure 5E**).

Association Between TP53 Mutation and Breast Cancer Immunogenicity

To determine how the TP53 mutation influences BC immunogenicity, we compared the expression differences in immune-related genes and stromal-related genes between the TP53-mutant group and TP53-wt group. The results indicated that in the mutation group, the levels of the ImmuneScore and StromalScore were both significantly increased (ImmuneScore: $p < 0.001$; StromalScore: $p = 0.003$; **Figure 6A**). Moreover, the expression of multiple HLA gene families was significantly upregulated in the mutation group (**Figure 6B**). Next, we used CIBERSORT to evaluate the composition ratio of 22 immune cell types in each BC sample and the result showed individual differences (**Figure 6C**). We also compared the levels of 22 immune cells between the TP53-mutant group and TP53-wt group. The results demonstrated that the proportions of Tregs, T helper cells, and M0 type macrophages were significantly upregulated in the TP53-mutant group (**Figure 6D**, $p < 0.05$), whereas the proportion of resting $CD4^+$ T cell and M2-type macrophages was lower (**Figure 6D**, $p < 0.05$).

Association Between the TP53 Status and Clinical Outcomes

We also performed the Kaplan-Meier analysis to assess the prognostic significance of TSPOAP1-AS1 expression. In the

TABLE 4 | KEGG pathways enriched in TP53-mutant and TP53-wt groups by using GSEA analysis.

Name	Size	Enrichment Score	NES	*p* value	Leading edge
KEGG_RIBOSOME	87	0.946565	1.633661	1.00E-10	tags = 84%, list = 4%, signal = 81%
KEGG_FOCAL_ADHESION	199	0.835	1.468514	2.31E-09	tags = 32%, list = 7%, signal = 30%
KEGG_ECM_RECEPTOR_INTERACTION	83	0.88544	1.526534	1.46E-06	tags = 35%, list = 5%, signal = 33%
KEGG_ANTIGEN_PROCESSING_AND_PRESENTATION	80	0.869832	1.498648	7.79E-06	tags = 38%, list = 9%, signal = 34%
KEGG_REGULATION_OF_ACTIN_CYTOSKELETON	212	0.767001	1.350277	3.37E-05	tags = 37%, list = 14%, signal = 32%
KEGG_ARRHYTHMOGENIC_RIGHT_VENTRICULAR_CARDIOMYOPATHY_ARVC	74	0.852098	1.467021	0.000171	tags = 22%, list = 5%, signal = 21%
KEGG_VIRAL_MYOCARDITIS	68	0.861515	1.480648	0.000171	tags = 24%, list = 4%, signal = 23%
KEGG_PATHOGENIC_ESCHERICHIA_COLI_INFECTION	55	0.878604	1.504304	0.000189	tags = 36%, list = 4%, signal = 35%
KEGG_LEUKOCYTE_TRANSENDOTHELIAL_MIGRATION	115	0.799328	1.39047	0.000193	tags = 32%, list = 10%, signal = 29%
KEGG_DILATED_CARDIOMYOPATHY	90	0.81603	1.409257	0.000393	tags = 13%, list = 5%, signal = 13%

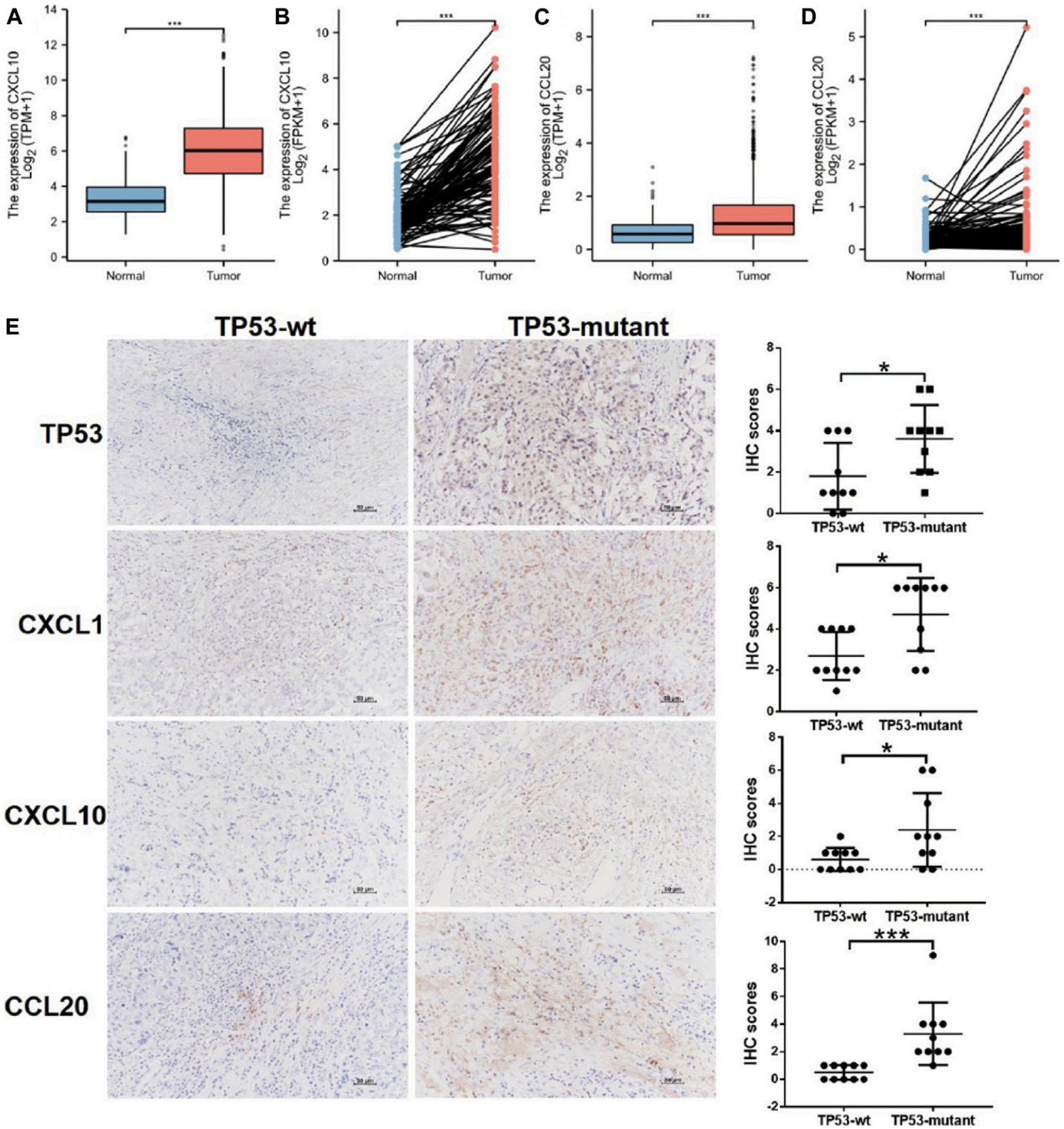

FIGURE 5 | Differential expression of CXCL1, CXCL10, CCL20, and TP53. **(A)** CXCL10 expression in BC tissues (n = 1109) vs. the normal tissues (n = 113). **(B)** CXCL10 expression in breast cancer tissues (n = 112) vs. the paired-paracancerous tissues (n = 112). **(C)** CCL20 expression in BC tissues (n = 1109) vs. the normal tissues (n = 113). **(D)** CCL20 expression in BC tissues (n = 112) vs. the paired-paracancerous tissues (n = 112). **(E)** IHC analysis of the expression of TP53, CXCL1, CXCL10, and CCL20 between TP53-wt (n = 10) and TP53-mutant (n = 10) group in BC tissues. BC, breast cancer.

TCGA-BRCA patients, the TP53 mutation was significantly associated with a shorter OS ($p = 0.038$; **Figure 7A**), whereas there was no significance for BRCA-FR ($p = 0.819$; **Figure 7B**) and BRCA-KR ($p = 0.301$; **Figure 7C**) patients. Then, to further confirm the prognostic value of the TP53 mutation, we conducted univariate and multivariate Cox regression analyses for OS. The results revealed that TP53 mutation ($p = 0.0298$), age ($p < 0.001$), tumor stage ($p < 0.001$), T stage ($p = 0.01$), N stage ($p < 0.001$), and M stage ($p < 0.001$) were correlated with BC prognosis (**Table 5**). Then, these variables were included to build

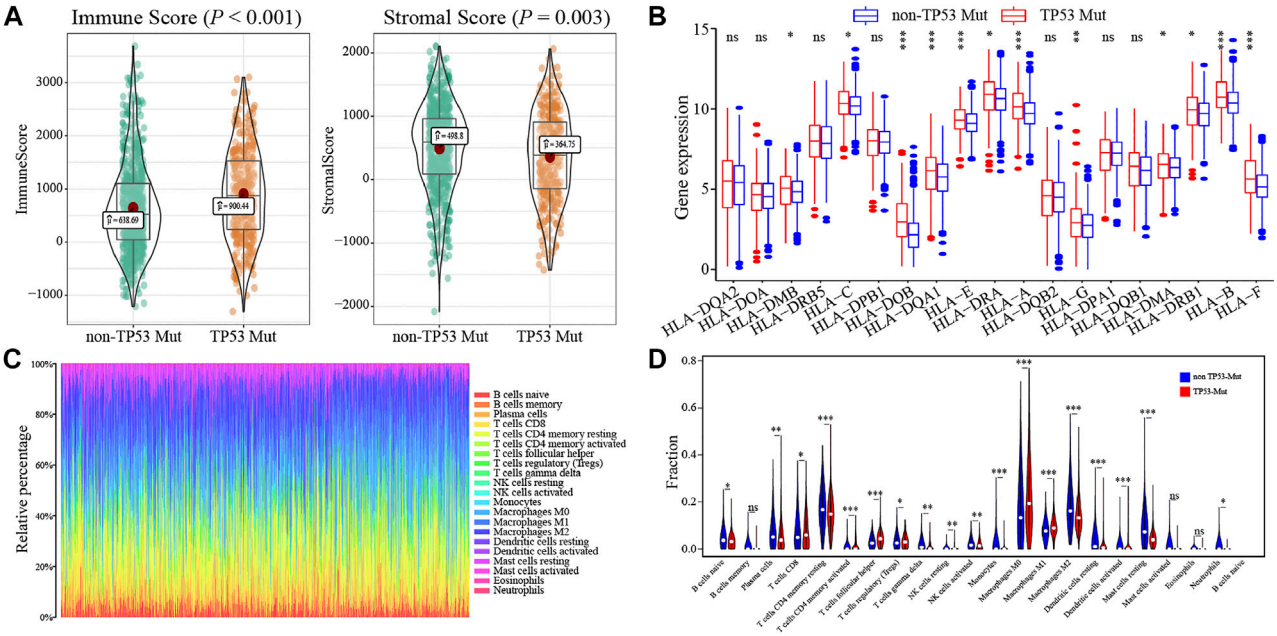

FIGURE 6 | The association between TP53 mutation and immunological phenotype in TCGA-BRCA patients. In TCGA-BRCA dataset, the ImmuneScore and StromalScore of TP53-mutant patients were significantly increased **(A)**; there was significant difference in the expression of HLA family genes between the two groups **(B)**; the histogram showed the proportion of 22 different immune cells in TCGA-BRCA patients **(C)**; the results of the difference analysis showed that the infiltration level of various immune cells was significantly different in TP53-mutant and TP53-wt group **(D)**.

the multivariable Cox models of OS (**Table 5**). The TP53 mutation remained independently associated with OS [HR: 1.76 (1.24–2.50), $p = 0.002$], which was also true for age [HR: 1.94 (1.37–2.76), $p < 0.001$], tumor stage [HR: 2.46 (1.26–4.80), $p = 0.009$], and M stage [HR: 1.67 (1.05–2.66), $p = 0.03$]. These results revealed that the TP53 mutation is an independent predictive factor of OS in BC patients. Further, to develop a clinical quantitative tool to predict the OS for BC patients, a nomogram was constructed based on the results of multivariable cox regression. In this nomogram, the significant variables including the TP53 mutation, age, stage, and TNM status were used to assign points (**Figure 7D**). The C-index of this nomogram was 0.772, and the calibration plots suggested that there was good consistency between the nomogram and observed OS probabilities in BC (**Figure 7E**).

DISCUSSION

TP53 mutations impair its capacity to bind the specific genome sequence that regulates the signaling pathway mediated by TP53 and lead to tumorigenesis and tumor progression in the context of other mutations present in the genome (Baugh et al., 2018). Prior studies revealed a role for TP53 in response to different treatments as complex as its different biological activities (Shahbandi et al., 2020). TP53 mutations contribute to the cancerous phenotype depending on the BC subtype (Silwal-Pandit et al., 2017; Schon and Tischkowitz, 2018). The patients with TP53 mutant tumors had worse survival than patients with TP53 wild-type tumors (Shahbandi et al., 2020). In luminal tumors, inactivation of TP53 via mutation causes the luminal B phenotype and resistance endocrine therapy, whereas mutant TP53 promotes epithelial-mesenchymal transition and stem cell properties in claudin-low and basal-like tumors (Coradini et al., 2012). Nevertheless, the barriers in understanding the clinical implications of TP53 mutations include an insufficient sample size and lack of long-term follow-up data for BC. Thus, we pooled the "Masked Somatic Mutation" datasets of 943 BC patients to analyze the characteristics and potential clinical significance of TP53 mutations, and the data was downloaded from TCGA GDC database. As a result, the TP53 mutation was prevalent in BC tissues and was an independent prognostic factor for poor prognosis. Moreover, we identified that TP53-mutant BCs presented with higher levels of immunogenicity including the ImmuneScore and StromalScore, and lower levels of TIDE than TP53-wt patients. Furthermore, patients with TP53 mutations tended to have richer immunocytes infiltration and more activated subsets in the TME compared to those in TP53-wt BC patients. These results indicated that ICI treatment is more effective in BC patients with TP53 mutations. Through further analysis, the possible mechanism through which TP53 mutations related to the efficacy of ICIs was determined to be its vital role in the tumor immune microenvironment.

We first explored the role of TP53 mutations in BC by assessing the correlation between the gene mutation and the response to immunotherapy in those patients. The results confirmed that the TP53-mutation group showed higher TMB ($p = 0.037$; **Figure 2C**) and MSI levels ($p = 0.077$; **Figure 2D**), which suggested that more

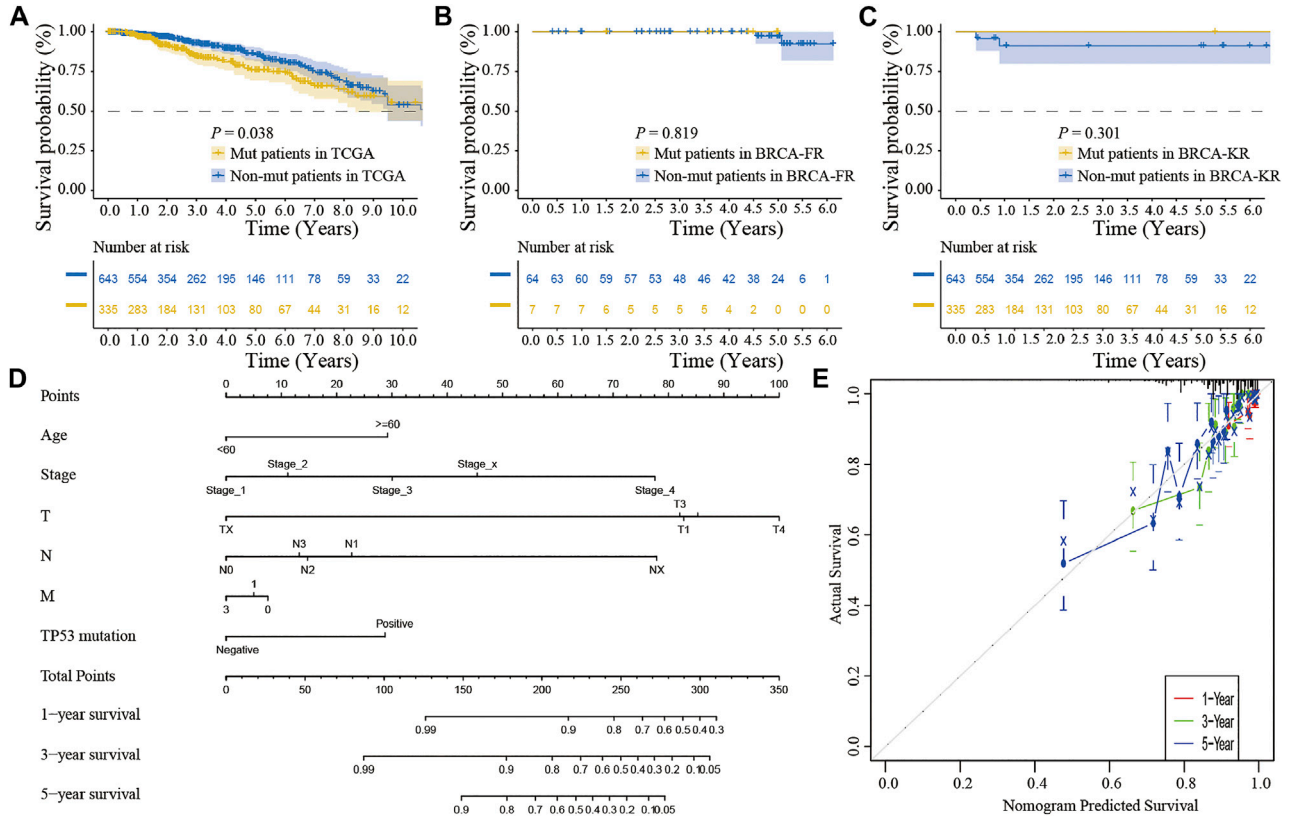

FIGURE 7 | The clinical significance of TP53 mutation in breast cancer patients. The survival analysis showed that the prognosis of patients with TP53 mutation was better in TCGA-BRCA dataset **(A)**; in BRCA-FR and BRCA-KR datasets, there was no significant correlation between TP53 mutation and disease prognosis **(B-C)**; the mutation of TP53 gene was combined with clinicopathological characteristics to construct the nomogram **(D)**; the calibration curve of the TP53 mutation was to evaluate the discrimination ability of the nomogram **(E)**. The horizontal coordinate was the survival predicted by the nomogram, and the vertical coordinate was the actual observed survival.

TABLE 5 | Association with overall survival and clinical pathologic characteristics using univariate and multivariate Cox regression.

	Univariate Cox analysis				Multivariate Cox analysis			
	HR	**HR.95L**	**HR.95H**	***p* value**	**HR**	**HR.95L**	**HR.95H**	***p* value**
Age (≥60 vs <60)	1.87	1.33	2.64	0.000324	1.94	1.37	2.76	0.000205
Stage (III + IV + X vs I + II)	2.50	1.77	3.53	1.91E-07	2.46	1.26	4.80	0.008534
T stage (T3 and T4 and TX vs T1andT2)	1.68	1.13	2.49	0.010329	0.87	0.51	1.50	0.627367
M stage (M1 and MX vs M0)	2.30	1.49	3.57	0.000177	1.67	1.05	2.66	0.031934
N stage (N2 and N3 and NX vs N0 and N1)	2.25	1.54	3.30	3.01E-05	1.06	0.57	1.96	0.855684
TP53-mutant (mutant vs. wt)	1.46	1.04	2.06	0.029765	1.76	1.24	2.50	0.001575

neoantigens could be recognized by endogenous immune cells, increasing cytotoxicity. This is also in accordance with the findings of previous research. Li et al. found that TP53-mutated cancers were more likely to have a higher level of tumor aneuploidy and TMB than TP53-wt cancers (Li et al., 2020). Moreover, based on the results of retrospective studies, TP53 mutation was found to be a potential biomarker for prognosis and efficacy prediction for BC (Duffy et al., 2018). Further, we also evaluated the TIDE, which is one of the important aspects of the tumor immune escape mechanism (Jiang et al., 2018). Surprisingly, TIDE was also significantly upregulated in the TP53-mutation group ($p < 0.001$; **Figure 2E**) compared with that in the WT group. This result suggests that the tumor microenvironment of TP53 mutated cancer cells might display an immune escape phenotype in BC. This might be because TP53 is the activator of apoptosis in response to DNA damage that functions by controlling tumor inflammation and immune response, and TP53 mutations could be used to reorganize the tumor immune composition (Blagih et al., 2020).

We further explored the correlation between TP53 status and the proportion of 22 immune cell subtypes in BC. By using the

CIBERSORT analysis package, we found that in the TP53-mutant group the proportion of Tregs, T helper cells, and M0 type macrophages was significantly upregulated, whereas the resting $CD4^+$ T cell and M2 type macrophages were downregulated. In the TME, TP53 regulates the balance between antigen-presenting cells and myeloid suppressor cells (such as Tregs), and the former could shape the anti-tumor immunity mediated by T cells. In addition, prior studies indicated that the TP53 mutation in tumors could modulate immune recognition by decreasing MHC-I presentation and increasing Treg recruitment (Bezzi et al., 2018). Meanwhile, TP53 mutations can also regulate $CD4^+$ T cells recruitment and their immune activity, thus leading to tumor cells escape from immune surveillance and promoting the tumor progression (Wellenstein et al., 2019). We also compared the BC immunogenicity differences between the TP53-mutant and TP53-wt groups. The results demonstrated that in the mutation group, the levels of the ImmuneScore ($p < 0.001$) and StromalScore ($p = 0.003$) were both significantly increased (**Figure 6A**). This result suggested that the TP53 mutation participated in modulating not only for the immune component, but also the stromal component of TME. Above all, TP53 plays a complex role in TME alterations by promoting the infiltration of diverse immunocytes, thus regulating the progression and prognosis of BC.

To explore the underlying mechanism, we compared differential expression in immune-related hub-genes between TP53-mutant and TP53-wt groups. The results demonstrated that the chemokines CXCL1, CXCL10, and CCL20 were significantly upregulated in the TP53-mutant group. Interestingly, we validated that the expression levels of CXCL1, CXCL10, and CCL20 increased in the TP53-mutant group ($p < 0.05$; Figure E). TP53 mutation modulated the production of cytokines and chemokines in cancer cells, which affect the proportion of immunocytes infiltrating the TME, including neutrophils, Tregs, and macrophages (Bezzi et al., 2018; Wellenstein et al., 2019). Previous findings demonstrated that tumor derived CXCL1 was expressed in stromal cells and epithelial cells, and promoted the cancer growth and its expression level related to the tumor grade (Addadi et al., 2010). Importantly, TP53 in CAFs relieves the repressive effect of chemokine CXCL1, thereby upregulating the migration and angiogenesis of tumor cells (Schauer et al., 2013). Further, macrophages were co-regulated based on TP53 and NF-κB signaling pathways, and TP53 was found to stimulate the secretion of CCL20 and CXCL1, which might facilitate tumor progression (Lowe et al., 2014). However, the TP53 mutation in macrophages either promotes the expression of the proinflammatory cytokines CXCL1 and CCL3, or eliminates the cells by initiating the apoptosis (Lowe et al., 2014). These changes might accelerate the malignant progression of cancer. In addition, the enrichment analysis results indicated that IL-17 signaling pathway was significantly altered in the TP53-mutant group (**Table 3**), which suggested that TP53 mutation might be involved in reorganizing the TME. Previous studies demonstrated that in BC IL-1β elicits IL-17 expression from γδT cells, and resulted in the polarization of neutrophils, yet the neutralization of IL-17 suppresses the T-cell-suppressive phenotype of neutrophils (Coffelt et al., 2015; Wu et al., 2020). Thus, IL-17 produced by neutrophils and γδT cells acts together to promote the metastasis of BC (Coffelt et al., 2015). These results illustrated that TP53-mutant BC cells were likely to promote the Treg infiltration into TME and secret more chemokines including CXCL1, CXCL10, and CCL20, contributing to several aspects of BC progression.

CONCLUSIONS

In summary, our findings indicate that the TP53 mutation is prevalent in BC and correlates with unfavorable prognosis. Meanwhile, TP53 mutation status is associated with different proportions of immunocytes infiltration, such as Tregs, $CD8^+$ T cells, and macrophages. Therefore, TP53 mutations have an essential influence on tumor immune microenvironment and provide a reference to further explore the effective immunotherapy for TP53-mutant BC.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

ZZ, RH, and XW designed the study and wrote the initial draft of the manuscript. ZZ, RH, QG, and SZ contributed to data analysis. ZZ, RH, and XW reviewed and edited the manuscript. All authors read and approved the manuscript.

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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.2021.759154/full#supplementary-material

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REVIEW
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Regulation of miRNAs Expression by Mutant p53 Gain of Function in Cancer

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The p53 roles have been largely described; among them, cell proliferation and apoptosis control are some of the best studied and understood. Interestingly, the mutations on the six hotspot sites within the region that encodes the DNA-binding domain of p53 give rise to other very different variants. The particular behavior of these variants led to consider p53 mutants as separate oncogene entities; that is, they do not retain wild type functions but acquire new ones, namely Gain-of-function p53 mutants. Furthermore, recent studies have revealed how p53 mutants regulate gene expression and exert oncogenic effects by unbalancing specific microRNAs (miRNAs) levels that provoke epithelial-mesenchymal transition, chemoresistance, and cell survival, among others. In this review, we discuss recent evidence of the crosstalk between miRNAs and mutants of p53, as well as the consequent cellular processes dysregulated.

Keywords: mutant p53, miRNAs, cancer, gain of function, miRNA biogenesis

INTRODUCTION

The miRNAs are short non-coding RNAs that function as post-transcriptional regulators of gene expression. (Jansson and Lund, 2012). Many miRNAs are found evolutionarily conserved in several organisms, which are suggested to have an important function in the essential biological processes (Jones and Lal, 2012). The miRNA biogenesis starts with the transcription of pri-miRNAs by RNA polymerase II from introns or exons of host genes but also from their promoters. The pri-miRNA forms a hairpin structure that is recognized and processed by the RNA binding protein Di George Syndrome (DGCR8) and a ribonuclease III enzyme (Drosha); the resulting product, a pre-miRNA is exported to the cytoplasm by exportin 5/RAN GTP complex. Within the cytoplasm, the pre-miRNA is cleaved by the rnase III endonuclease Dicer, giving rise to a mature miRNA duplex (Denli et al., 2004). The Argonaute family of proteins (AGO1-4) can be loaded with any strand of the miRNA duplex, but the strand with lower stability is more likely loaded into AGO (Ha and Kim, 2014). The complex formed by the guide strand and AGO is considered the minimal miRNA-induced silencing complex (miRISC). A perfect pairing between miRNA and its mRNA target induces rapid Poly(a)-deadenylation and decapping steps, which cleavage and degrade the mRNA target. However, most interactions are not fully complementary; therefore, the miRISC complex in the first place interferes with the translation initiation (Li et al., 2014a; O'Brien et al.,

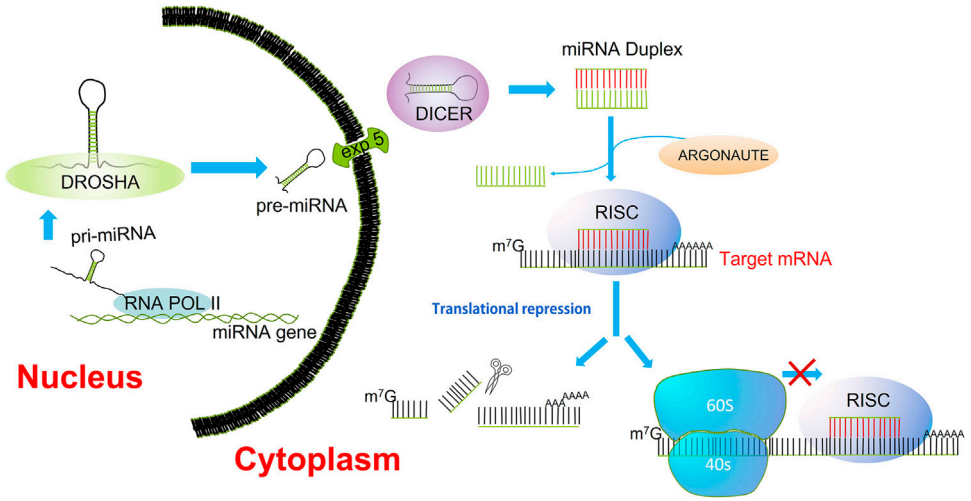

FIGURE 1 | miRNA biogenesis. Transcription of miRNAs is mediated by RNA polymerase II. The pri-miRNAs are substrates for the rnase III, Drosha. The product of pre-miRNA cleavage by Drosha is exported to the cytoplasm by the nuclear transporter exportin 5. In the cytoplasm, DICER processes the pre-miRNA into a miRNA/miRNA* duplex. Final processing yields the mature miRNA duplex, the strand with lower thermodynamic stability is loaded into Argonaute (AGO); this is deemed the guide strand. The guide strand alongside AGO constitutes the minimal RNA-induced silencing complex (RISC). Once recruited the RISC complex onto the mRNA target, the inhibition of translation is carried out through cleavage, deadenylation, or blocking of the mRNA translation.

2018) (**Figure 1**). Moreover, a given mRNA can be concurrently regulated by multiple miRNAs, and an estimated 60% of the human genome is regulated by miRNAs (Hermeking, 2012).

miRNAs are regulators of numerous cellular processes, including proliferation, differentiation, and apoptosis (Hainaut and Hollstein, 2000; Hermeking, 2012). Many miRNAs map to specific regions of the human genome frequently deleted or enhanced in human cancers (Croce, 2009). Growing evidence demonstrates that they form unique expression patterns or signatures (Calin and Croce, 2006; Donzelli et al., 2014). Interestingly, it has been reported that p53, the most frequently mutated gene in human cancer, modulates miRNA expression (Hermeking, 2012; Jones and Lal, 2012; Li et al., 2014a). The mutations in the gene *TP53* are mainly missense, resulting in the production of a full-length mutant p53 protein, unlike most tumor suppressor genes inactivated through biallelic deletion or truncation mutations (Li et al., 2014a; Muller and Vousden, 2014). The mutations of p53 are frequently in six 'hotspot' residues within the DNA-binding domain: R175, G245, R248, R249, R273, and R282 (Hainaut and Hollstein, 2000; Mello and Attardi, 2013). Because these missense mutations are mainly located in the DNA-binding domain of p53, the mutant p53 protein is unable to transactivate most of its target genes resulting in a loss of the protein p53 wild type function. Besides, the mutant p53 protein can, in many cases, lose its functions and may exert a dominant negative regulation on any remaining p53 wt (Petitjean et al., 2007).

Moreover, mutant p53 also acquires oncogenic functions that modulate various phenotypes such as epithelial-mesenchymal transition (EMT), migration, invasion, metastasis, chemoresistance, proliferation, apoptosis and genomic instability (Li et al., 2014a); these functions are entirely independent of p53 wt function (Strano et al., 2007; Brosh and Rotter, 2009). The mutations of p53 can be divided into two main classes: 1) DNA contact defective mutants, whose residue subjected to mutation is located in the region of the protein that binds to DNA (R273H, R273C, R248Q, and R248W) and 2) Structural defective mutants, whose mutation impairs a residue critical for the entire folding of the protein (R175H, G245S, R249S, and R282H) (Bullock and Fersht, 2001). In addition, recent studies have shown that mutant p53 can regulate gene expression and exert oncogenic effects through specific miRNAs (Li et al., 2014a).

Here, we review the mechanisms by which mutant p53 gains diverse oncogenic functions by regulating specific miRNAs.

REGULATION OF DIVERSE CELLULAR PROCESSES THROUGH MIRNAS BY MUTANT P53

Cell Cycle and Apoptosis

miR-517a

The miR-517a fulfills physiological roles associated with the progression of pregnancy (Miura et al., 2010). It has also exhibited opposite functions regarding cellular proliferation or tumor suppressor activities. For instance, Yoshitomi and others observed that in bladder cancer cell lines, the overexpression of miR-517a led to inhibition of cell proliferation and increased cell apoptosis (Yoshitomi et al., 2011). Moreover, knockdown of miR-517a in glioma cells led to diminished cell proliferation and higher apoptosis; then, using a cell-line-derived xenograft mouse model, the U87 glioma cells lines expressing sh-miR-517a, showed lesser tumor growth as compared to the wild type cells (Du et al., 2019). Likewise, the silencing of miR-517a in melanoma cells induced up-regulation of CDKN1C (inhibitor of

cyclin/cyclin-dependent kinase complexes), cleaved caspase-3, Bax/Bcl2 ratio, as well as high levels of Reactive Oxidative Species (ROS) and diminished cell proliferation (Yang et al., 2020). It is important to note that these reports do not clarify how miR-517a induces the aforementioned effects; meanwhile, other authors have been approaching identifying some of its targets. For instance, in SW48 and HCT116 colon cancer cells, an inverse correlation was identified between the expression of miR-517a and the forkhead box J3 (*FOXJ3*) tumor suppressor (Ma et al., 2016).

The expression of this and other microRNAs was assessed in the context of mutants of p53. Recently, Garibaldi and others carried out a genome wide expression analysis of 376 mature miRNA in SW480 mutant (p53R273H/P309S) colon cancer cells before and after constitutive depletion of the endogenous mutant p53. This analysis showed that mutant p53R273H downregulates 33 and upregulates four of 376 miRNAs (Garibaldi et al., 2016a). Among the data obtained, they observed that miR-520g, miR-518b, miR-582, miR-141, miR-519c, miR-143, miR-142-3p, and 142-5 were upregulated both at pri-miRNA and pre-miRNA levels after mutant p53 depletion, suggesting regulation at transcriptional level. Moreover, miR-517a, miR-519a, miR-105, miR-628, miR-1, miR-218, miR-515-3p, and miR-515-5p showed no significant change in primary transcripts after mutant p53 depletion, which was possibly due to a post-transcriptional regulation. After that, the authors demonstrated a post-transcriptional regulation of miRNAs through the interaction of mutp53 and the Drosha complex. This finding was corroborated by co-immunoprecipitation, confocal analysis, and RNA-chromatin immunoprecipitation experiments, which determined that endogenous mutant p53R273H directly binds to RNA helicases p72/82, hindering the association of this DEAD-box with other members of the microprocessor complex (Drosha), resulting in the inhibition of the miRNA biogenesis (Garibaldi et al., 2016a).

Although the overall biogenesis of miRNAs was hampered by mutant p53, one of the most downregulated miRNAs by the mutant p53R273H and p53R175H was miR-517a. In this regard, previous studies reported that miR-517a inhibits cell proliferation by blocking the G_2/M transition in hepatocellular carcinoma cells (Liu et al., 2013). The underlying mechanism may involve the proline-rich tyrosine kinase 2 (Pyk2), which is a target of miR-517a; Pyk2 was shown to promote cell proliferation and invasiveness by upregulation of the c-Src and ERK/MAPK signaling pathways in hepatocellular carcinoma cells (Sun et al., 2008). Consistent with this, Garibaldi et al. demonstrated that the ectopic expression of the miR-517a impaired the SW480 cell survival as indicated by the reduction of viable cell number, the increase of trypan blue-positive cells, and by a diminished colony-forming ability, suggesting that this miRNA restrain the cell cycle progression. In addition, by cytofluorimetric assays, it was observed that miR-517a induced cell cycle arrest in G_2/M, and induced apoptosis, which was determined by caspase activation, induction of cleaved PARP, and Annexin V assays (Garibaldi et al., 2016a).

Altogether, these functions of miR-517a pinpoint its important role as a tumor suppressor that is negatively regulated by the mutant p53R273H in colon cancer cells (**Figure 2**; **Table 1**).

miR-27a

miR-27a have a pivotal role in multiple processes, including cancer development, osteogenesis (Gu et al., 2016; You et al., 2016) adipogenesis (Kim et al., 2010; Hu et al., 2018), cell proliferation (Xu et al., 2013; Su et al., 2019), apoptosis (Sun et al., 2019) and differentiation (Kim et al., 2010; Tang et al., 2014).

Wang and others performed a custom miRNA microarray analysis to compare the miRNA expression profiles between p53 wt and mutant p53 in H1299 cells. The authors employed a p53-inducible system where the addition of doxycycline triggered the expression of p53 wt (H1299-Tet-On-p53) or mutant p53R273H (H1299-Tet-On-p53-273H). Mutant p53R273H exhibited differential expression of multiple miRNAs as compared with p53 wt. Among these findings, miR-27a was remarkably downregulated and furtherly studied. In addition to p53R273H, the mutants p53R175H and p53G279E, but not the wild-type p53, exhibited inhibitory effects on miR-27a expression. By chromatin immunoprecipitation (ChIP) assays, the authors determined a strong binding of mutant p53R273H with the miR-27a promoter within the nucleotide 2,899 to 2,675, indicating that this p53 mutant transcriptionally downregulates miR-27a. Next, the authors identified the epidermal growth factor receptor (*EGFR*) gene as a direct target of miR-27a; therefore, as p53R273H suppresses the expression of miR-27a, the *EGFR* expression increases, which leads to cell proliferation and tumor growth (Wang et al., 2013) (**Figure 3A**; **Table 1**). Concerning EGFR, some inhibitors that target this receptor's kinase domain (TKIs) have been developed to tackle its permanent activation in patients with non-small cell lung cancer (Landi and Cappuzzo, 2013; Russo et al., 2017; Masood et al., 2019). Therefore, it would be interesting to analyze the response to TKIs among patients that harbor p53 mutations, especially those with lung cancer where the *EGFR* can be found overexpressed.

miR-105

miR-105 and other miRNAs exhibit opposite roles since they can behave like an oncogene or like a tumor suppressor. This miRNA was found upregulated in esophageal cancer tissues and was associated with positive lymph node metastasis, advanced TNM stage, and poor overall survival (Gao et al., 2020a). Conversely, miR-105 was downregulated in gastric cancer tissues, and its overexpression inhibited cell migration, invasion, and EMT in gastric cancer by targeting *SOX9* (Shang et al., 2019). In accordance, another report showed that up-regulation of miR-105 suppressed the colony formation and aggressiveness traits of gastric carcinoma cell lines BGC823 and SGC7901 *in vitro*. In this report, the authors also identified *SOX9* as the target of miR-105 (Jin et al., 2019). Since the role of miR-105 can apparently be interplayed as a tumor suppressor or as an oncogene, it would be difficult to bet for a certain gene therapy targeted to this microRNA.

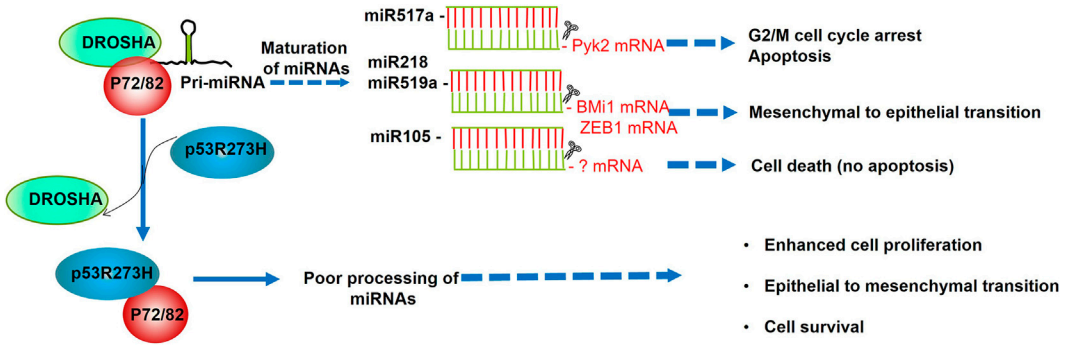

FIGURE 2 | p53R273H promotes cell survival, proliferation, and epithelial to mesenchymal transition through sequestering p72/82. The association of p72/82, members of the DEAD-box family of RNA helicases, with DROSHA, is necessary to achieve a fine-tune processing of specific subsets of miRNAs. As p53R273H sequesters RNA helicases p72/82 from the microprocessor complex, this interferes with the maturation of miR517a, miR218, miR519a, and miR105. According to the biological processes regulated by these miRNAs, their downregulation provokes higher cell proliferation, cell survival, and EMT promotion.

TABLE 1 | miRNAs Regulated by mutant p53.

Mutant of p53	*miRNA*	*Process*	*Reference*
p53R175H	miR-128-2	Cell cycle and apoptosis	Donzelli et al. (2012)
p53R175H	miR-223	Cell cycle and apoptosis	Masciarelli et al. (2014)
p53R273H	let-7i	Migration, Invasion and Metastasis	Subramanian et al. (2015)
p53R273H	miR-27a	Cell Proliferation	Wang et al. (2013)
p53G279E			
p53R175H			
p53R273H, p53R248Q, p53R175H, *p53C135Y*	miR-130b	Promote Epithelial Mesenchymal Transition	Dong et al. (2013)
p53R248Q, p53R282W, *p53R249S*	miR-155	Migration, Invasion, and Metastasis	Neilsen et al. (2013)
p53R273H, p53R175H	miR-517a	Cell cycle and apoptosis	Garibaldi et al. (2016a)
p53R273H	miR-519a	Promote Epithelial Mesenchymal Transition	Garibaldi et al. (2016a)
p53R273H	miR-105	Cell proliferation	Garibaldi et al. (2016a)
p53R273H	miR-218	Promote Epithelial Mesenchymal Transition	Garibaldi et al. (2016a)

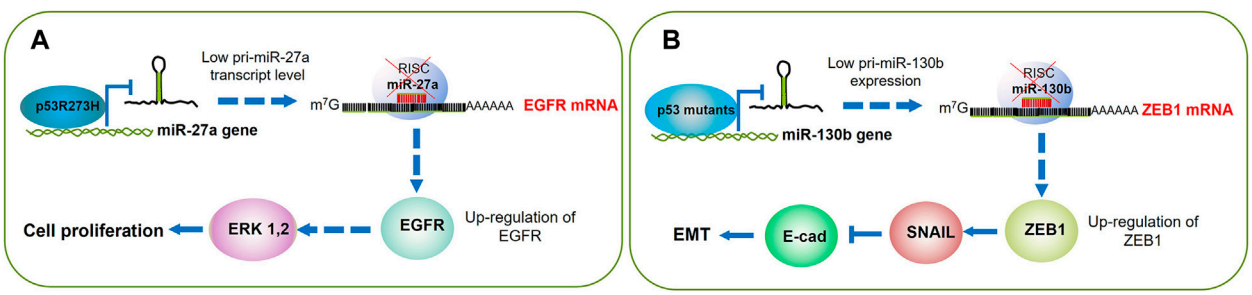

FIGURE 3 | p53 mutants boost cell proliferation and EMT through gene promoter silencing. **(A)** p53R175H binds to and silences the miR-27a gene promoter; this leads to higher expression of its EGFR target. Then EGFR triggers the cell signaling that results in ERK 1,2 activation and cell cycle progression. **(B)** The mutants p53R175H, p53R273H, P53C135Y, and P53R248Q turn off miR130b expression, which results in the accumulation of ZEB1, that in turn activates SNAIL and consequent silencing of E-cadherin; altogether leading to activation of the EMT program.

Regarding mutant p53, the p53R273H impaired the expression of miR-105; the diminished level of this miRNA provoked high cell proliferation and low apoptosis. Conversely, when miR-105 was transiently transfected in SW480 cells, the cell proliferation did not change, but the cell death was evident (Garibaldi et al., 2016a). It is worth noting that the cell death was not apoptotic, and it remains to clarify what kind of cell death induces the miR-105 (Figure 2; **Table 1**).

It would be important to analyze whether the presence of other p53 mutations affects the expression of miR-105, also in other types of cancer, and whether it has or not a prognostic value.

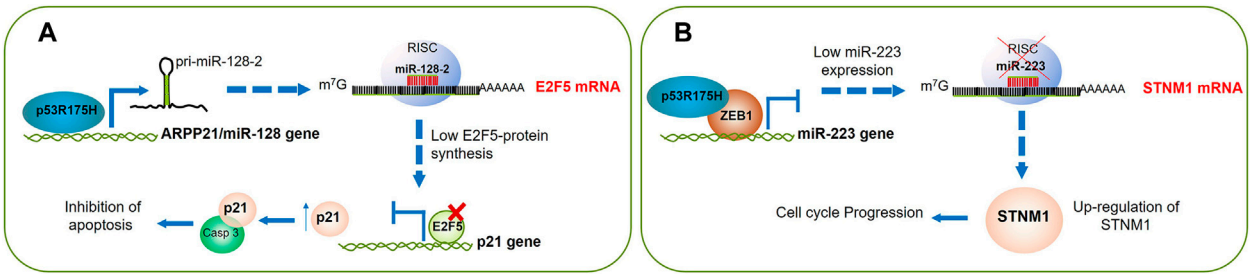

FIGURE 4 | The cell cycle progression and inhibition of apoptosis can be triggered by p53R175H. **(A)** Mutant p53R175H induces miR-128-2 expression whose direct target is E2F5 mRNA. Downregulation of E2F5 blocks its repressive activity on p21 transcription, then p21 protein is accumulated in the cytoplasm, where it exerts an anti-apoptotic effect by binding to pro-caspase-3. **(B)** ZEB1 binds to miR-223 gene promoter and alongside p53R175H both inhibit miR-223 transcription, this allows increasing synthesis of STNM1 that is involved in chemoresistance and cell cycle progression.

Chemoresistance and Cell Survival

miR-128-2

miR-128 is an intronic miRNA encoded by two different genes, miR-128-1 and miR-128-2, both located within the introns of *R3HDM1* and *ARPP21* genes, respectively (Li et al., 2013). It is considered that the function of miR-128 depends on the cellular context; however, it is mainly associated with the development and maintenance of the nervous system. Besides, it also has clear roles in the context of tumor cells as it was observed downregulated in glioblastoma, prostate cancer, lung cancer, colorectal, and breast cancer (Huang et al., 2015).

miR-128-3-p was used to enhance the chemosensitivity of colorectal cancer cells. This micro RNA was packed in a complex with PEG-PDMAEMA (Poly (ethylene glycol)–poly (2-(dimethylamino)ethyl methacrylate) that was following decorated with a peptide that targets the monocarboxylate transporter1 (tumor-homing peptide). This nanocomplex successfully delivered the micro RNA within cancer cells and inhibited the PI3K/AKT and MEK/ERK pathways. In combination with 5-Fluorouracil (5-FU), this nanocomplex dramatically improved the chemotherapy effects (Liu et al., 2020). In another interesting work, the authors used the intestinal Fetal Human Cells (FHC)for packing miR-128-3p within exosomes that were later exposed to oxaliplatin-resistant colorectal cancer cells. The authors observed a remarkable improvement in the oxaliplatin response and up-regulation of E-cadherin and reduced EMT (Liu et al., 2019).

Regarding p53 mutants, Donzelli and others searched for differential expression of a battery of miRNAs commonly altered in lung cancer in response to p53 mutants. Interestingly, using the H1299 cells, a human non-small-cell lung cancer (NSCLC) cell line, carrying a ponasterone (Pon-A) inducible mutant p53R175H protein; they found that the expression of intragenic miR-128-2 increases upon mutant p53R175H protein induction. Their studies demonstrated that the mutant p53 binds to the putative promoter of the miR-128-2 host gene, *ARPP21*, determining a concomitant induction of ARPP21 mRNA and miR-128-2 (Calin and Croce, 2006; Donzelli et al., 2012). To further investigate the contribution of miR-128-2 modulation to mutant p53 gain of function activity, they assessed whether miR-128-2 exogenous expression impacts the response of H1299 lung cancer cell line to doxorubicin (DOXO), cisplatinum (CDDP), and 5-fluorouracil (5-FU). They observed that miR-128-2 expression confers chemoresistance to all of these drugs. According to the experimental evidence, the authors further demonstrated that miR-128-2 post-transcriptionally targets E2F5, leading to the abrogation of its repressive activity on p21 transcription. Although nuclear p21 is strongly associated with growth arrest, it was observed that when p21 protein localizes to the cytoplasmic compartment, it exerts an anti-apoptotic effect by preventing pro-caspase three cleavage. Therefore, this study highlights the role of miRNA-128-2 on chemoresistance by inhibition of apoptosis in NSCLC (Donzelli et al., 2012) (**Figure 4A**; **Table 1**). Otherwise, considering that micro-RNAs potentially target more than one gene, it is quite possible that miR-128-2 may foster chemoresistance through the silencing of other genes different than *E2F5*. It would be also interesting to demonstrate whether other mutants of p53 can regulate the miR-128-2 expression; if this is the case, then it could also be a helpful marker to predict chemoresistance at least in NSCLC; yet, this effect is apparently context-dependent because, in colorectal cancer, glioblastoma, and ovarian cancer, the overexpression of miR-128-2 has been associated with chemosensitivity (Li et al., 2014b; She et al., 2014; Lian et al., 2018).

miR-223

miR-223 is located within the q12 locus of the X chromosome, and it is known to be an important factor for the differentiation and function of the immune system (Ye et al., 2018; Zhou et al., 2018). Regarding cancer, miR-223 has shown opposite roles because it has been observed upregulated in acute lymphoblastic leukemia and bladder cancer but downregulated in chronic lymphoid leukemia and hepatocellular carcinoma (Zhou et al., 2018). When miR-223 was overexpressed in HCT116 cells, the cell proliferation was increased, and the apoptosis diminished. These effects were presumably a consequence of the inhibition of the *FBXW7* tumor suppressor by miR-223 (Liu et al., 2021). Similarly, another study reported that miR-223 enhanced chemoresistance to cisplatin in human non-small cell lung cancer cells. The suggested underlying

mechanism was the downregulation of *FBXW7* and subsequent activation of autophagy (Wang et al., 2020). In contrast, in breast cancer tumors, miR-223 was lost in the early stages, and its absence caused resistance to CDK4/6 inhibitors (Citron et al., 2020).

On the other hand, Donzelli (Donzelli et al., 2012), Masciarelli and others observed miR-223 downregulated upon expression of p53R175H (Masciarelli et al., 2014). To further investigate this finding in a more physiological context, they silenced the mutant p53 in SW480 (colon cancer cell line) or MDA-MB-468 and MDA-MB-231 cells (breast cancer cell lines), resulting in an increased miR-223 expression (Masciarelli et al., 2014). By chromatin immunoprecipitation (ChIP) assays, they provided evidence showing that p53 was bound to the miR-223 regulatory region. Interestingly, the authors also identified ZEB1 as an important transcription factor that cooperates with mutant p53 for the silencing of miR-223. Through ChIP-reChIP assays, they observed direct binding between mutant p53 and ZEB1 on the miR-223 regulatory region, concluding that mutant p53 does not bind directly to the DNA, but it does through ZEB1. The authors identified the miR-223 STMN1 target, a key microtubule-regulatory protein essential for cell cycle progression (Cimmino et al., 2005; Saito et al., 2015), as responsible for chemoresistance. *STMN1* was previously found downregulated by p53 wt (Ahn et al., 1999); it was associated with recurrence, metastasis, and drug resistance, and indeed, it has been studied as a therapeutic target (Rana et al., 2008; Phadke et al., 2011). In accordance, Masciarelli and others determined that silencing of *STMN1* in the mutant p53 SW480 cell line increased cell death in the presence of DNA damage induced by cisplatin. They also found that silencing of *STMN1* was associated with an inhibition of Cdk1 activity that provokes an arrest in Mitosis. Altogether, these data suggest that mutant p53R175H downregulated miR-223, which in turn upregulates *STMN1* expression leading to cancer cell resistance to chemotherapy in colon and breast cancer cell lines (Masciarelli et al., 2014). To further validate these findings, it will be important to determine if the presence of mutant p53R175H, p53R273H, or p53R280K and/or overexpression of *STMN1* are associated with chemoresistance in patients with colon and breast cancer; if this association is confirmed, it might be a valuable chemoresistance prognostic factor (**Figure 4B**; **Table 1**).

Epithelial-Mesenchymal Transition (EMT)

miR-130b

miR-130b has been found either up or downregulated in several cancers; then, it can function as an oncomiR or as a tumor suppressor. On the one hand, miR-130b-3p was highly expressed in nephroblastoma, and it was inversely correlated with the expression of phosphatase and tensin homolog (PTEN) protein (Hu and Yan, 2019). Another study showed miR-130b-3p upregulated in neoplastic versus normal prostate tissue, even in mestastatic versus primary sites (Fort et al., 2018). Hirono and others. found miR-130b significantly increased in NSCLC specimens from patients with vascular and lymphatic invasion (Hirono et al., 2019). Altogether these reports agree miR-130b exhibits oncogenic roles. However, another report showed the opposite. Zhao and others observed miR-130b downregulated in 52 pancreatic cancer tissues and 5 cell lines. Moreover, as they overexpressed miR-130b, the proliferation of pancreatic cancer cells was dramatically suppressed both *in vivo* and *in vitro*; similarly, miR-130b remarkably diminished the invasivity of pancreatic cancer cells (Zhao et al., 2013).

Regarding invasion and metastasis driven by EMT, Dong and others found an interesting connection with p53 GOF mutants. These authors carried out an array-based miRNA profiling of p53-null HEC-50 endometrial cells transduced with three mutants, p53R273H, p53R175H, and p53C135Y or empty vector; 23 out 188 miRNAs were expressed above background levels. The authors observed an inverse correlation between miR-130-b and these p53 mutants, plus p53R248Q. In turn, the silencing of the mutant p53R248Q in HEC-1 cells led to the upregulation of miR-130b. To determine whether the endogenous p53 mutant can bind to the promoter of miR-130b, chromatin immunoprecipitation (ChIP) and qPCR analysis on HEC-1 cells was made, demonstrated that miR-130b is a direct target of p53 mutant in endometrial cancer (EC) cells.

The *in silic*o analysis predicted binding of miR-130-b to the 3′UTR of ZEB1 mRNA, and such interaction was further demonstrated in HEC50 and HEC-1 cells resulting in the repression of ZEB1. Given the outstanding capability of this transcription factor to promote invasion and metastasis by inducing EMT (Zhang et al., 2015), the authors provided evidence of the induction of metastatic-associated genes such as SPP1, *MMP2*, and *MMP9* as well as EMT-promoting genes like *ZEB1*, *BMI1*, *CDH2* (N-cadherin) and *VIM* (Vimentin) in the presence of p53 mutants (Dong et al., 2013).

These data demonstrate that p53 GOF mutants downregulate miR-130b expression, which results in activation of *ZEB1*, and its downstream pathway contributes to the induction of EMT and increased EC cell invasion (Dong et al., 2013) (**Figure 4B**; **Table 1**).

miR-218 and miR-519a

As reported by Garibaldi and colleaagues, the mutant p53R273H downregulated miR-218 and miR-519a in colon cancer cells (Garibaldi et al., 2016a). Moreover, the overexpression of these miRNAs impairing the migratory capability of SW480 cells was determined by wound-healing assays. Interestingly, the presence of these miRNAs was associated with a lower level of *Zeb1* and upregulation of *CDH1* (E-cadherin); the *in silico* analysis indicated one putative binding site for miR-218 and two sites for miR-519a on the 3' UTR of *ZEB1*. In agreement, another report showed that miR-218 diminished the invasion and metastasis of gastric cancer by suppressing the Robo1 receptor, which activates the slit-Robo1 pathway triggering metastasis *in vivo* and *in vitro* (Tie et al., 2010). Similarly, when miR-218 was restored in glioma cells, cell migration, invasion, and proliferation dramatically reduced, presumably by targeting the stem cell promoting oncogene *BMi1* (Tu et al., 2013).

Regarding miR-519a, its expression was markedly diminished in cancer tissues from the ovary, lung, and kidney, and this was

correlated with high levels of the RNA-binding protein HuR. In turn, when miR-519 was overexpressed in human cervical carcinoma xenografts, the tumor size was significantly smaller than controls (Abdelmohsen et al., 2010). Altogether, the evidence strongly suggests that mutants of p53 can promote EMT of tumor cells by inhibiting miR-218 or miR-519a (**Figure 2**; **Table 1**).

Let-7i

The function of the let7 family members is rather versatile. In the vertebrates, the let-7 family, along with miR-100 and miR-125, form a complex system of developmental regulators (Hertel et al., 2012). Besides, the let-7 miRNAs perform diverse functions such as regulators of cell proliferation, cell cycle, migration, progression, stem cell biology, metabolism, and chemoresistance (Mizuno et al., 2018; Chirshev et al., 2019). Some of the let-7 miRNA family members in the brain have been shown to serve as ligands of Toll-like receptors (TLR7) expressed in the microglia. Interestingly, the resulting signaling induces inflammatory cytokines microglial release that modulates antigen presentation and reduces cell migration. Furthermore, the expression of let-7 miRNAs also inhibited the growth of murine GL261 glioma through microglial TLR7 (Buonfiglioli et al., 2019). In accordance, the let-7 miRNAs have been found altered not only in glioblastomas but in an extensive list of cancers such as breast, ovarian, pancreatic, lung, liver, gastric and oral squamous cell carcinoma, among other types of cancers (for an excellent review see Boyerinas and others. 2010) (Boyerinas et al., 2010).

The p53 mutants can influence the let-7 miRNAs expression as well. Subramanian and others sequenced small RNAs from the p53-null H1299 lung cancer cell line, stably transfected with the hotspot aggressive mutant p53R273H (32). In this approach, they observed 38 miRNAs up- and three downregulated; the tumor suppressor let-7i was abundant in the control cells but significantly down-regulated in H1299 cells expressing p53R273H. Stable knockdown of endogenous mutant p53 in MDA-MB-231 (breast cancer, p53R280K) and DLD1 (colorectal cancer, p53S241F) cell lines released the let-7i repression. After that, by ChIP assays from MDA-MB-231 cells, the authors determined that while mutant p53 inhibits the occupancy of p63 on the let-7i promoter, the mutant p53-p63 complex is bound to the let-7i promoter as well, both mechanisms working together to silence the let-7i expression. Interestingly, the breast cancer MDA-MB-231 and pancreatic cancer MIA-PaCa-2 xenografts showed minor migration and invasion when let-7i was introduced. Then, the authors identified the targets of let-7i by microarray assays and validated them by qRT-PCR; these were mainly oncogenes like *E2F5*, *HMGA1*, *MYC*, and *N-RAS*, and genes related to RNA metabolism like *CPSF1*, *DDX18*, and *LIN28B* (Subramanian et al., 2015).

The transcendental finding of this work is perhaps the reaffirmation of the concept that the mutants of p53 exert a dominant negative effect over their wild type counterpart and further to the other members of the p53 family, in this case, the p63 protein; the outcome of this interaction, at least in this report, turned out in cellular proliferation, migration, and invasion (**Figure 5A**; **Table 1**).

miR-155

miR-155 is a master regulator of the immune system. It is expressed mainly in the thymus and spleen and in lesser extension in other tissues (Mashima, 2015). It was originally identified as a gene B-cell integration cluster (bic), considered a proto-oncogene in chickens, then it was realized that miR-155 is expressed from bic exon 2 (Vigorito et al., 2013).

The expression of miR-155 in the immune cells such as macrophages or dendritic cells, leads to cytokines positive regulation. miR-155 can modulate activated myeloid and lymphoid cells transcriptome that influences several biological functions such as inflammation or immunological memory (Vigorito et al., 2013). Regarding cancer, this micro-RNA has shown a protagonist role. miR-155 is correlated with poor prognosis in patients with bladder cancer (Wang and Men, 2015); its expression, along with miR-27a, promoted tumor metastasis and chemoresistance in gastric cancer (Li et al., 2020). Although miR-155 is majorly identified as an oncomiR, its overexpression prevented the cell migration and invasion of HT-29 gastric cancer cells (Liu et al., 2018).

Interestingly, Nielsen and others showed the interconnection between mutants of p53 and miR-155 and their role in promoting cell migration and invasion. Using a scratch-wound assay, they first observed an enhanced ability to migrate the ZR-75-1 and MCF10A epithelial breast cancer cells, both stably transfected with miR-155. Moreover, the expression of miR-155 was tightly associated with the presence of mutants of p53, since the induced expression of either p53R248Q or p53R282W in the p53-null H1299 cells was associated with a dose-dependent increased expression of mature miR-155 levels while knockdown of endogenous mutant p53 in BT-549 (p53R249S) down-regulated miR-155, suggesting that miR-155 is a mutant p53 target in breast cancer cells.

As mentioned before, some mutants of p53 are known to impair the function of p63. By ChIP experiments, the authors demonstrated that in MCF-10A cells, the endogenous p63 was directly recruited to consensus p63-Response Elements, localized within the promoter of the miR-155HG microRNA host gene, in the absence of mutant p53; this finding suggests that p63 represses miR-155 and that this repression is canceled by mutant p53; however, they did not demonstrate a direct association between mutants of p53 and p63 to support this mechanism.

By performing a thorough analysis in the literature, the authors identified 42 candidate genes that drive invasion in breast tumors, downregulated by mutant p53 presumably through miR-155. From this list, four genes (*ZNF652*, *PDCD4*, *TCF12*, and *IL17RB*) were downregulated in tumors with the highest frequency of metastasis-related poor outcomes, being *ZNF652* the best candidate for further investigation since it was previously demonstrated to be downregulated by miR-155. The *ZNF652* gene, encodes for a transcription repressor factor (Kumar et al., 2006; Kumar et al., 2008), which was shown to be directly recruited to gene regulatory elements of *TGFB1*, *TGFB2,*

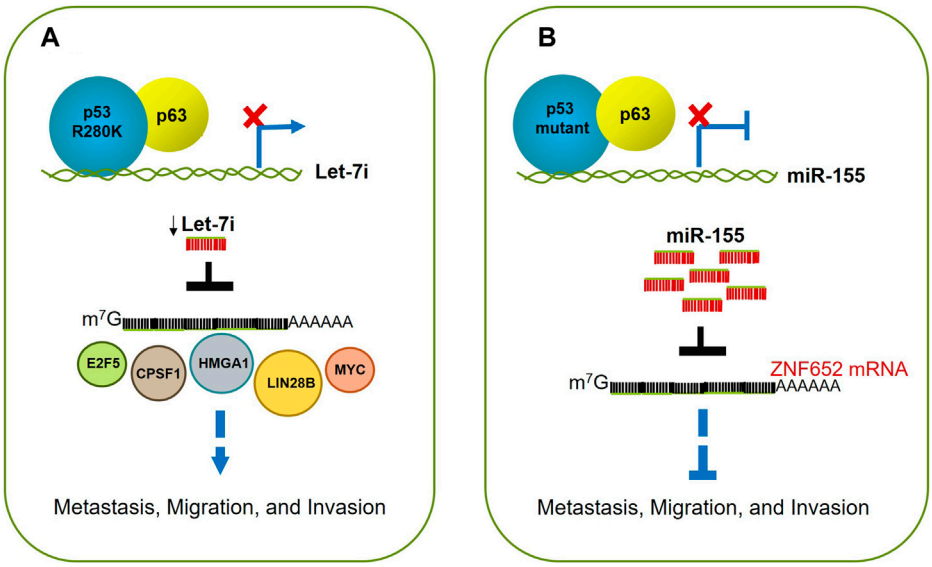

FIGURE 5 | p53 mutants drive migration, invasion, and metastasis. **(A)** p53R280K associates with p63 blocking its occupancy on let-7i promoter; this leads to downregulation of let7i, and increasing levels of its targets like E2F5, CPSF1, HMGA1, LIN28B, MYC, and NRAS. The alteration of this balance fuels cell proliferation, migration, and invasion. **(B)** Mutant p53 (p53R248Q or p53R282W) acquires enhanced invasive and metastatic potential through up-regulation of miR-155. This oncomiR promotes invasion by directly repressing the target transcript ZNF652, which as a consequence, causes the acquisition of an invasive cell phenotype.

TGFBR2, EGFR, SMAD2, and *VIM* through ChIP analysis in ZR-74-1 cells. Besides, the silencing of *ZNF652* led to the upregulation of these genes, indicating that this gene is indeed required for their repression. To acquire more knowledge about the extent of *ZNF652* influence, the authors performed gene expression profiling in the absence of *ZNF652* or overexpressing miR-155. They observed a correlation between the EMT gene expression program in the absence of *ZNF652* or the presence of miR-155, both converging within the TGF-β signaling pathway (Neilsen et al., 2013). Accordingly, the *ZNF652* levels were lower in malignant invasive breast tumors in comparison with normal breast tissue.

Altogether, these findings support the hypothesis that mutants of p53 can promote invasion and metastasis because of the up-regulation of miR-155. In turn, this oncomiR triggers the EMT program, involving the TGF-β pathway, through the repression of the tumor suppressor *ZNF652* (Neilsen et al., 2013) (**Figure 5B**; **Table 1**).

Vesicular Secretion

The extracellular vesicles are involved in cell-to-cell communication. The cargo can be lipids, proteins, or nucleic acids. In a cancer scenario, the cancer cells-delivered vesicles are uptaken by the tumor microenvironment cells, which can foster the tumor progression, metastasis or drug resistance (Maleki et al., 2021). One of the most overexpressed microRNA in cancer-derived exosomes is the miR-21. It was found that this oncomiR is transferred from cisplatin-resistant oral squamous carcinoma cells through exosomes, and this transference conferred resistance to cisplatin-sensitive cells by targeting *PTEN* and *PCD4* (Liu et al., 2017). Previously, it was observed an association between abundant expression of miR-21 and human metastatic tumors harboring p53 mutations (Bornachea et al., 2012). After that, other researchers linked the overexpression of miR-21 with specific mutations in p53 (R175H and R248Q); however, the overexpression of this oncomiR is just a piece of the landscape since p53 mutants, as described below, can also remodel the extracellular matrix and improve vesicle secretion thus fostering cell migration and drug resistance.

miR-30d

It has been recently demonstrated that p53 mutants (R175H, R273H, and R280K) can induce structural changes in the Golgi Apparatus. These changes were mainly related to an increase in the number of *cis*-Golgi elements and a replacement of perinuclear ribbon-like structure by multiple mini-stacks dispersed within the cytoplasm. In addition, the p53 mutant-induced Golgi remodelation increased notably the vesicular trafficking and secretion. The underlying mechanism seems to imply that p53 mutants form a complex with HIF1-alfa that transactivate miR-30d; then, this miRNA represses *AP2A1, DGK2, PPP3CB,* and *VPS26B*, all associated with vesicular trafficking and recycling processes.

This increasing secretion provoked some interesting changes in the extracellular matrix (ECM). By atomic force microscopy, the authors observed that p53 mutants enhance the ECM rigidity; then, after analyzing the content of proteins secreted by mutant p53-knockdown in MDA-MB231 cells, a significant decrease in the levels of fibronectin, laminin V, and laminin B1 was observed. It is not surprising that all these changes led to the alteration in cell migration. When H1299 cells were exposed to a medium

collected from MDA-MB231 cells, their migration capability increased more than two-fold. Accordingly, when immunodeficient mice were injected with luciferase-expressing MDA-MB-231 cells defective in the expression of miR-30d, the tumor growth was delayed over a period of 4 weeks, and notably, the lung colonization was dramatically reduced too (Capaci et al., 2020). As expected, the use of the drug PRIMA-1MET, which restores p53 wt function, resulted in the downregulation of miR-30d in MDA-MB231 cells.

Considering that this mechanism involves vesicular secretion, it would be interesting to explore whether the use of drugs that affect this route may impair the p53 mutant-induced vesicle trafficking and exosome secretion. In fact, some drugs like anti-malarial chloroquine and its derivative hydroxychloroquine are widely accepted for their ability to impair autophagy (Mauthe et al., 2018). Yet, their capability to de-acidify lysosomes and Golgi apparatus also preclude the vesicular secretion (Halcrow et al., 2021). Then a combinatorial therapeutic scheme seems to be a good choice in the appropriate context, that is, in the presence of p53 mutants. Altogether, these authors provided interesting evidence that showed how p53 mutants increase the vesicular trafficking and secretion, which favors the generation of a microenvironment permissive for metastatic colonization (Capaci et al., 2020).

Furthermore, the p53 mutants could facilitate the delivery of vesicles charged of oncomiRs through this recently described mechanism. For instance, it was observed that miR-105 can be transferred via exosomes from huvec cells; the resulting impact on the uptaking cells was the loss of *Z O -1* expression and thereby the loss of the epithelial architecture and polarity (Zhou et al., 2014). In accordance, it was observed that the co-culture of macrophages with gastric cancer cells or ovarian cancer cells led to an induction of chemoresistance in both cell lines. The underlying mechanism involved the uptake of macrophage-derived exosomes enriched with miR-223 by the gastric- and ovarian cancer cells (Zhu et al., 2019; Gao et al., 2020b). Since it was not specified the status of p53 in the macrophage THP1 cells, it remains to explore whether p53 mutants improve the exosome secretion. Also, it is unclear if the mutant-p53 inducing overexpression of miR-21 in oral squamous cancer cells is also linked to its vesicular packing and secretion.

REGULATION OF MIRNA BIOGENESIS BY MUTANT P53

So far, the knowledge about the underlying misregulation of miRNAs in cancer, rather than mutations or deletions, seems to be oriented to an imbalance of miRNA levels by impaired maturation at pri- or pre-miRNA processing steps (Calin and Croce, 2006). For an excellent review about miRNA biogenesis in the context of mutants of p53, see the manuscript of Aymone Gurtner and collaborators (Gurtner et al., 2016). For instance, by using the p53 wild-type HCT116 human colon cancer cell line, Suzuki and others determined that p53 wt can enhance the post-transcriptional maturation of several miRNAs with a growth-suppressive function such as miR-143 and miR-16-1 that target K-Ras, and miR-145 which targets CDK6 in response to DNA damage. The authors observed that p53 wt interacts with the Drosha complex through p68/p72 helicases; the carboxy-terminal half of the DNA-binding domain of p53 is the interacting region with p68 or Drosha complex. On the contrary, the p53 mutants R273H, R175H, and C135Y interact in a lesser extension with p68, leading to attenuation of miRNA processing activity (Suzuki et al., 2009).

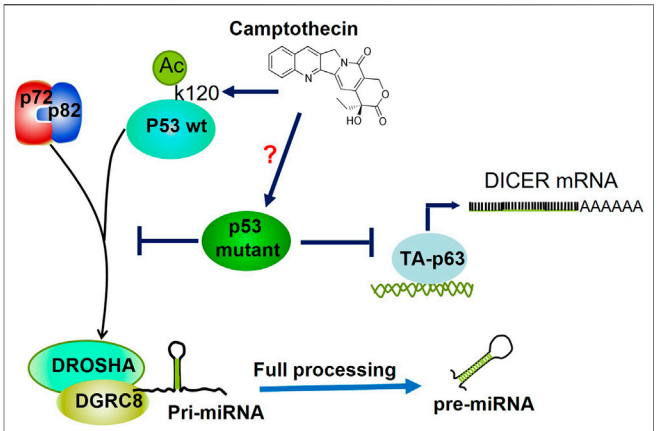

FIGURE 6 | Regulation of miRNAs processing by Mutant p53. Mutants of p53 hinder the association between RNA helicases p72/82 and the microprocessor complex (DROSHA-DGCR8), thereby inhibiting miRNAs' maturation post-transcriptional level. Camptothecin induces acetylation of K120 within the DNA Binding Domain of p53, which augments its association with p72/p82. Whether this drug may revert the poor association of p53 mutants with p72/p82 is not known. p53R273H inhibits the transcription of DICER mRNA through both direct and indirect inhibition of transactivation of TA-p63.

Similarly, Chang and others observed that camptothectin-induced acetylation in lysine 120 (K120) within the DNA-binding domain of p53 promoted its association with the Drosha complex leading to improved processing of miR-203. However, augmenting the maturation of miR-203 blocks Bcl-2, thus inducing the p53-dependent cell death (Chang et al., 2013). Since K120 is not one of the p53 hot spot mutations, its acetylation on p53 mutants via camptothectin would be an interesting approach to observe whether this drug may revert the affinity loss towards Drosha observed in p53 mutants (**Figure 6**).

Following these findings, Jiang and others reported that mutants of p53 (C135Y, R175H, R248Q, and R273H) promoted EMT in endometrial carcinoma (EC) a similar mechanism. As opposite to p53 wt, the mutants of p53 induced poor processing and maturation of pri-miR-26a-1 since its respective pre-miR-26a-1 mature form was diminished in the presence of the mutants of p53. The lack of maturation resulted from a loss of interaction between the Drosha complex and p68 induced by these mutants. The target of miR-26a is Enhancer of Zeste Homolog 2 (*EZH2*), the catalytic subunit of the Polycomb Repressive Complex that is involved in EMT and is best known to repress a large number of tumor suppressor genes. Stepwise, the authors showed that

overexpression of miR-126a reverted the EMT phenotype in HEC-1B cells (Jiang et al., 2015).

As mentioned previously, Garibaldi and others demonstrated that endogenous mutant p53R273H binds to and sequesters RNA heliscases p72/82, thus interfering with its association with Drosha and therefore precluding pri-miRNAs processing and biogenesis. In agreement, they determined that p53-R175H and -R280K can interact with p72/82 through its N-terminal domain. Since mutant p53 does not bind to pri-miRNAs, then p72/82 complex recruitment is pri-miRNA independent. Moreover, the analysis of pri-miRNA, pre-miRNA, and mature miRNA expression shows that mutant p53 downregulates miRNAs at the transcriptional and post-transcriptional levels. Consistent with this, p53wt has an opposite effect on the expression of mutant p53-repressed miRNAs on colon cancer cell lines (Garibaldi et al., 2016b). Besides, Muller and others delineate a clear relationship between mutant p53, p63, and Dicer that might contribute to the metastatic function of mutant p53. They showed that mutant p53 can downregulate Dicer expression through both direct and indirect inhibition of the TAp63-mediated transcriptional activation of Dicer. Consistent with this, the transient expression of mutant p53R273H in H1299 caused a decrease in endogenous Dicer mRNA and protein expression (Muller et al., 2014) (**Figure 6**).

In an integrative effort, another study involved the use of multi-omics technologies to identify the common targets among five mutants of p53, such as p53R175H and p53R273H, in triple negative breast cancer cell lines (TNBC), the study showed that proteasome machinery is particularly overactivated. The proteasome activity targets for degradation of some tumor suppressors like p21, p27, and *NOXA*; interestingly, it also targets KSRP, which is crucial for the maturation of microRNAs. Even more interesting was the counteracting effect of the co-treatment of carfilzomib (a proteasome inhibitor) and PRIMA-1MET (a mutant p53-inactivating agent) on TNBC cells that resulted in a markedly diminished chemoresistance (Walerych et al., 2016; Di Agostino, 2020).

CONCLUDING REMARKS

For a while, it has been shown the characteristic capabilities of p53 mutants not just for losing their wild type functions but also for acquiring new ones. Among these properties, their ability to modulate both coding and non-coding RNAs is outstanding. The astonishing fact that some miRNAs behave differently according to the cellular context opens a new research path to explore; one possible explanation may come from observations of Vasudevan and others. Strikingly, they observed that microRNAs could make a switch that activates the protein expression depending on the cellular environment instead of silencing a target gene (Vasudevan et al., 2007). Specifically, when cells were under serum starvation, they became quiescent, and in this state, they exhibited upregulation of a reporter gene. The authors determined that AGO-2 (Argonaute) and FXR1 (Fragile X mental retardation-related protein 1) are involved in the miRNA switch only in cells under stress but not in proliferating unstressed cells. This fact could aid in explaining that under certain circumstances or stress conditions, the microRNAs change their role from silencers to translation inducers. In a cancer scenario, where hypoxia or nutrient starvation are common events, it could trigger the converse response of microRNAs. Whether p53 mutants regulate the cellular environment to influence this condition is unknown; for instance, the expression of FXR1 is found upregulated in colorectal cancer (Jin et al., 2016). Then, it would be interesting to search if p53 mutants can upregulate this oncogene. Another plausible explanation for this duality may be due to the diverse and wide number of targets that one miRNA can have. In other words, one miRNA can target several oncogenic mRNAs and several tumor suppressive mRNAs simultaneously, so the balance of both may dictaminate the final cell fate. For instance, miR-125 can silence a plethora of mRNAs whose function is opposed to each other. For example, it targets antiapoptotic factors (BCL2, BCL2L2, MUC1), proapoptotic factors (TP53, BAK1, PUMA), metastatic factors (MMP1, VEGFA, ERBB2/3) among others (Sun et al., 2013). Hence, miR-125 have shown diverse facets, as an oncomiR in most hematologic malignancies and as a tumor suppressor in solid tumors (Svoronos et al., 2016). Another contrasting behavior is presented by miR-155. As we mentioned above in the "EMT section", this miRNA is associated with mutant p53 expression and also is correlated with EMT and migration in breast cancer cells (**Figure 5B**); however, under ionizing radiotherapy miR-155 favour an apoptotic outcome by targeting RAD51 (Gasparini et al., 2014), which is a critical factor for the DNA repair thorugh homologous recombination. The more affected the DNA repair machinery is, the more susceptible the cell is under DNA damage, which turns out in better overall survival**.** Given that miRNAs regulate approximately 60% of the global expression of genes, it is not surprising the growing number of reports that associate a determined miRNA profile with a specific disease. The mutants of p53 can impinge the maturation of miRNAs, resulting in the imbalance of miRNAs levels in some types of cancers, thus producing a specific miRNAs expression profile. In fact, Zhang and others in 2016 identified a miRNA signature that correlated with poor cancer outcomes. Using a microarray method, they observed the miRNAs either up-or downregulated in response to the expression of the p53mutant R282W in H1299 cells. Once analyzed by the non-negative matrix factorization model and Kaplan-Meier test, these data revealed a mutant-p53 signature identifying cancer subgroups with significantly different outcomes. These observations were reproducible for liver, breast and gastric cancer (Zhang et al., 2016). In another report, there were examined 121 patients with HNSCC to explore the status of p53. From these cohorts of patients, it was identified a signature of 12 miRNAs showed an association with shorter recurrence-free survival among those that harbored p53 mutations (58%) (Ganci et al., 2013; Valenti et al., 2019).

In addition, several studies show that patients with tumors carrying p53 mutations have shorter survival, worse prognosis and poorer response to conventional anti-cancer treatments. In order to tackle this, scientists are developing several drugs

targeting the mutations already discussed in this issue; then, it would be very interesting to assess whether these drugs may revert the altered expression of miRNAs provoked by mutants of p53. Besides, the recently described modes of action of p53 mutants also open new treatment alternatives, specifically by tackling the secretory pathway. In this regard, chloroquine, hydroxychloroquine and Ivermectin are promise drugs that have a great anti-cancer potential. With this knowledge, this therapy hopefully could be more focused among patients harboring p53 mutations.

There remain some open questions, for instance, given that p53 mutants impair the function of factors that regulate the overall maturation machinery of microRNAs, which is perhaps the reason that p53 mutants induce an overall dowregulation of microRNAs, then we wonder ¿how do the mutants of p53 do for up-regulating specific microRNAs? or ¿how do they do to down-regulate some microRNAs in a quite larger extension than others?

In summary, the full comprehension of the molecular events underlying gain of function of mutant p53 proteins is essential for improving our understanding of the acquisition of aggressive traits of carcinoma cells, such as invasion and metastasis. It thus may help us to propose new target therapies.

AUTHOR CONTRIBUTIONS

J-DC contributed to the conception and design of the review. TM and JH-M wrote the first draft of the manuscript. LH, CR, GD-G, ML-M, and KG wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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GLOSSARY

WT wild-type
UTR untranslated region
p53 tumor suppressor protein
TP53 tumor protein 53 gene
miRNA short non-coding RNAs
RISC silencing complex
AGO2 argonaute 2
TRBP TAR RNA binding protein
PACT double strand dsRNA-binding protein
ChIP chromatin immunoprecipitation
EGFR epidermal growth factor receptor
ERK1/2 mitogen-activated protein kinases
ARPP21 cAMP-regulated phosphoprotein 21
DOXO doxorubicin
CDDP cisplatinum
5-FU 5-fluorouracil
E2F5 transcription factor 5
p21 cyclin-dependent kinase inhibitor
ZEB1 zinc finger E-box binding homeobox 1
STMN1 stathmin 1
Pyk2 pyruvate kinase
PARP poly-(ADP-ribose) polymerase
GOF gain-of-functions of mutant p53
IP propidium iodide
MMP-2 matrix metallopeptidase 2
MMP-9 matrix metallopeptidase 9
BMI1 polycomb ring finger oncogene
HuR RNA binding protein
EV empty vector
CPSF1 cleavage and polyadenylation specific factor
HMGA1 high mobility group AT-hook 1
LIN28B lin-28 homolog B
MYC v-myc avian myelocytomatosis viral oncogene homolog
NRAS neuroblastoma RAS viral oncogene homolog
DDX18 DEAD-box helicase 18
DDX17 DEAD-box helicase 17
DDX5 DEAD-box helicase 5
TGF-β transforming growth factor
p63 tumor suppressor protein
p63-RE p63 response element
ZNF652 zinc finger protein 652
PDCD4 programmed cell death 4
TCF12 transcription factor 12
IL17RB interleukin 17 receptor B
SMAD2 SMAD family member 2
DGCR8 DiGeorge critical region 8
Cdk1 cyclin dependent kinase 1
MAPK mitogen activated kinase-like protein
EGF epidermal growth factor
Robo1 roundabout 1
TGFB1 transforming growth factor beta 1
TGFB2 transforming growth factor beta 2
TGFBR2 transforming growth factor beta receptor 2
TAp63 p63 isoform.

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PERSPECTIVE
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Rab11-FIP1/RCP Functions as a Major Signalling Hub in the Oncogenic Roles of Mutant p53 in Cancer

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Rab11-FIP1 is a Rab effector protein that is involved in endosomal recycling and trafficking of various molecules throughout the endocytic compartments of the cell. The consequence of this can be increased secretion or increased membrane expression of those molecules. In general, expression of Rab11-FIP1 coincides with more tumourigenic and metastatic cell behaviour. Rab11-FIP1 can work in concert with oncogenes such as mutant p53, but has also been speculated to be an oncogene in its own right. In this perspective, we will discuss and speculate upon our observations that mutant p53 promotes Rab11-FIP1 function to not only promote invasive behaviour, but also chemoresistance by regulating a multitude of different proteins.

Keywords: Rab11-FIP1, RCP, p53, recycling, cancer, integrin, invasion, metastasis

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INTRODUCTION

Rab11-FIP1 was identified as a downstream effector and interactor of the Rab-GTPase Rab11a, important in membrane recycling systems (1). Rab GTPases form a family of more than 70 members, regulating vesicle trafficking in different cell localisations or compartments (2–4), and cycle between a membrane-bound state (bound to GTP) and a cytosolic state (free of GTP). Rab11 specifically, has been shown active and involved in endocytosis, recycling compartments and the trans-golgi network, regulating endocytic membrane traffic. When bound to GTP, Rab11 interacts with Rab11-FIP1 in the early endosomal recycling compartment (5). Rab11-FIP1 is required for endosomal recycling, and regulates the sorting of proteins into endosomes and the delivery of cargo to the plasma membrane (6, 7). Its cargo can be diverse and includes receptor tyrosine kinases,

Abbreviations: Akt, Protein Kinase B; ATP7B, Copper-transporting ATPase 2; DGK- α, Diacylglycerol Kinase alpha; EGF/EGFR, Epidermal Growth Factor (Receptor); FAK, Focal Adhesion Kinase 1; FHOD3, Formin Homology 2 Domain Containing 3; HGF/HGFR, Hepatocyte Growth Factor (Receptor); IGF2R, Insulin Like Growth Factor 2 Receptor; LMTK3, Lemur Tyrosine Kinase 3; MARK2, Microtubule Affinity Regulating Kinase 2; MT1-MMP, Membrane-Type 1 Matrix Metalloproteinase; P-gp, P-glycoprotein; PDGFR, Platelet-Derived Growth Factor Receptor; Rab11-FIP1, Rab11 Family-Interacting Protein 1; RCP, Rab Coupling Protein; ROCK, Rho-associated Protein Kinase; RTK, Receptor Tyrosine Kinase; TfnR, Transferrin Receptor; VEGFR, Vascular Endothelial Growth Factor Receptor.

integrins and other membrane receptors or molecules schematically depicted in **Figure 1** and discussed below.

Based on the frequent overexpression of Rab11-FIP1 in cancers (overexpression or amplified 8p11–12 amplicon), Rab11-FIP1 was proposed to be an oncogene (8, 9). Importantly, Rab11-FIP1 can drive metastasis *in vivo*, which was demonstrated using a pancreatic ductal adenocarcinoma mouse model harbouring pancreas-specific p53 and K-Ras mutations. In this context, loss of Rab11-FIP1 reduced the overall metastatic burden (10). In contrast, some data suggest that loss of Rab11-FIP1 promotes oncogenesis or invasion in cervical or oesophageal cancers (11, 12). Rab11-FIP1 can therefore not be classed as an oncogene in its own right yet. It is possible that increased Rab11-FIP1 function or expression is context-dependent and enhanced by the presence of oncogenes, including mutant p53, or by a tumour promoting environment in which cytokines, integrins and growth factors such as EGF are enriched (13).

MUTANT p53 AND THE ROLE OF RAB11-FIP1 IN INTEGRIN/RTK SIGNALLING

p53 is a transcription factor involved in many different processes, including cell death and senescence. By reacting to incoming stresses, p53 activates specific transcriptional programmes, such as apoptosis or cell cycle arrest. This allows the organism to stop further accumulation of DNA damage and allows for DNA repair or cell death depending on the amount of stress.

Mutations in *TP53* can lead to loss of p53 protein expression or in about 75% of cases, to the expression of a mutant p53 protein (14). Mutant p53 expression results in loss of the tumour suppressor function as well as acquisition of a gain-of-function that promotes proliferation, invasion, metastasis or chemoresistance. Proposed mechanisms for gain-of-function include binding to new response elements on the DNA and the interaction with many different proteins, including transcription factors such as the p53 family member p63 (15). We have shown previously that mutant p53 inhibits TAp63α function, and in those same conditions, mutant p53 promotes the interaction between integrins, RTKs and the Rab-coupling protein/Rab11-Family Interacting Protein 1 (RCP/Rab11-FIP1), leading to increased invasion, cell scattering, metastasis and chemoresistance (16–18).

Integrins form a family of glycoprotein cell surface receptors that interact with the microenvironment. By binding to extracellular ligands, they promote adhesion to the extracellular matrix, other cells or activate intracellular signalling pathways that are shared and interconnected with receptor tyrosine kinases (RTKs) (19). Integrins are thus involved in a range of cellular processes that promote tumorigenesis and confer a survival advantage to cancer cells. RTKs are cell surface receptors that bind to growth factors, hormones and cytokines to promote cell signalling mediated through their inherent tyrosine kinase activity. The integrin/RTK cooperation amplifies signalling, promoting tumour formation, aggressiveness and drug resistance.

We and others have shown that the cooperation of integrin β1 and EGFR is dependent on Rab11-FIP1 (13, 16, 20, 21). In some cells, this was dependent on stimulation with the EGFR ligand EGF, or with the αvβ3 integrin ligand osteopontin (13). However, when mutant p53 was expressed, osteopontin activation was not required to induce Rab11-FIP1-dependent delivery of integrins and EGFR to the plasma membrane (16). Downstream of EGFR and integrins, the Akt/PKB pathway and its substrate RacGAP1 were activated (22). Activation of RacGap1 lead to the repression of cytoskeleton regulator Rac1, promotion of RhoA activity and cytoskeleton re-organisation to extend pseudopodial protrusions with actin spikes, leading to increased invasion (22). These actin spike extensions depended on activation of the protein FHOD3 by ROCK (23). Part of the role of Rab11-FIP1 in the pseudopodia is dependent on diacylglycerol kinase α (DGK-α) (24). DGK-α phosphorylates diacylglycerol to phosphatidic acid (25). Rab11-FIP1 can interact with phosphatidic acid resulting in the mobilisation of Rab11-FIP1 into the pseudopodia of the cells.

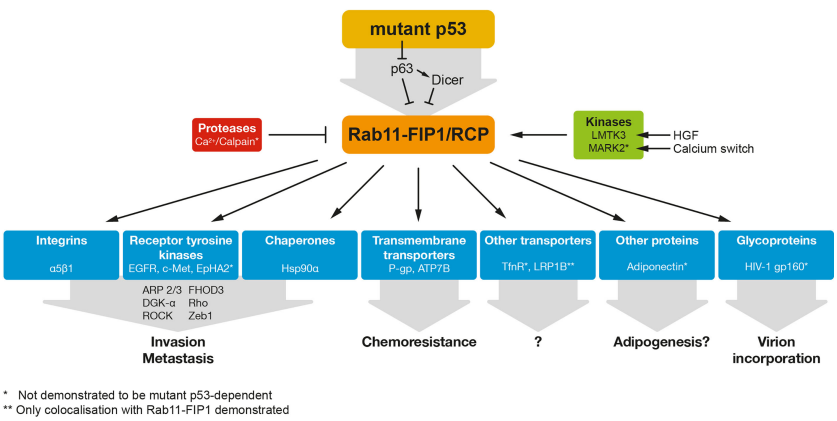

FIGURE 1 | Mutant p53 regulates RAB11-FIP1-dependent re-localisation of a variety of proteins. Mutant p53 can regulate RAB11-FIP1 by inhibiting the p53 family member p63 and/or the downstream target Dicer. Rab11-FIP1 enhances the re-localisation of a variety of proteins indicated in this figure. * indicates mutant p53 dependency. ** indicates only co-localisation demonstrated with Rab11-FIP1.

Beside regulating EGFR, we have more recently demonstrated that mutant p53 also promotes the association of Rab11-FIP1 with c-Met (HGFR, hepatocyte growth factor receptor) (26). This interaction promoted the scattering of cells and increased pERK1/2 signalling, contributing to HGF-mediated invasion. To a large extent, all these interactions were dependent on the p53 family member TAp63α and its target gene, the microRNA machinery protein Dicer (27). Combined integrin-RTK signalling is thought to amplify signalling to the Erk1/2 and Akt proteins, driving enhanced invasion, cell scattering and metastasis (16, 17).

As we demonstrated that mutant p53 through Rab11-FIP1 could promote recycling of both EGFR and c-Met, it is tempting to consider that other RTKs are regulated in this manner. Likely candidates could be EphA2, IGF2R, PDGFR and VEGFR. EphA2 has been shown to bind to Rab11-FIP1 to mediate metastasis *in vivo*. In response to HGF, Akt phosphorylates EphA2. In parallel, HGF promotes phosphorylation of Rab11-FIP1 by the Lemur tyrosine kinase-3 (LMTK3) leading to EphA2 binding and plasma membrane expression. This results in cell-cell repulsion, driving metastatic behaviour (10). However, an involvement of mutant p53 was not demonstrated and remains to be elucidated. IGF2R, PDGFR and VEGFR have all been found transcriptionally regulated by GOF mutant p53 (28–31), although a connection with Rab11-FIP1 has not yet been established. Perhaps by both regulating expression as well as actual plasma membrane expression, something that is likely also occurring for β1 integrin (32), mutant p53 could facilitate an amplified cell signalling response that promotes metastastic behaviour and causes the multidrug chemoresistance that is often seen in mutant p53 tumours.

Taken together, these data demonstrate that mutant p53 can regulate integrin/RTK signalling in a Rab11-FIP1-dependent manner to promote invasion and metastasis.

MUTANT p53, RAB11, RAB11-FIP1 AND OTHER MECHANISMS PROMOTING INVASION AND METASTASIS

Interestingly, mutant p53 was also shown to promote invasion and metastasis by modifying the secretome of cells in a Rab11-FIP1-dependent manner (33, 34). Novo et al. demonstrated that mutant p53 cells with upregulated Rab11-FIP1 are able to influence the phenotype of distant cells through the production of podocalyxin-containing exosomes. These exosomes then remodel the extracellular matrix, supporting invasion of the mutant p53 cells through upregulated Rab11-FIP1 and, notably, α5β1 integrin, c-Met and Transferrin Receptor (TfnR) recycling in neighbouring wildtype p53 or p53-null cells (33).

Additionally, Zhang et al. propose a model in which mutant p53 promotes the vesicular trafficking and secretion of the Hsp90α chaperone in a Rab11-FIP1 dependent manner (35) (**Figure 1**). HSP90α secretion occurs at least in some tumours and cancer cell lines (36–38) and is known to promote tumorigenesis (39–41). The Hsp90α interaction with extracellular matrix proteins and receptors is thought to underlie matrix remodelling and increased invasion and metastasis of mutant p53 cells (35).

In conclusion, these data suggest that Rab11-FIP1 can also act over longer distances through cargo exosome secretion to promote invasion and metastasis.

MUTANT p53 AND RAB11-FIP1 PROMOTE CHEMORESISTANCE

It has been shown that enhanced integrin signalling confers resistance against several chemotherapeutic compounds (42–44). In cultured lung adenocarcinoma A549 cells, Rab11-FIP1-mediated β1 integrin recycling and signalling was able to confer resistance to cisplatin (45). Various others have shown that increased activation of RTKs *via* integrins confers chemoresistance through RTK signalling (44, 46). As mutant p53 promotes chemoresistance (47–49), it therefore seemed likely that mutant p53 could promote chemoresistance through the Rab11-FIP1/integrin/EGFR signalling pathway. Indeed, cells in which we knocked-out Rab11-FIP1 appeared to become more sensitive to etoposide and cisplatin (18). However, when inhibiting integrins, the sensitivity was less pronounced compared to loss of Rab11-FIP1 expression, suggesting alternative pathways are involved in this chemoresistance (18).

Interestingly, in a screen to detect novel Rab11-FIP1 interacting proteins, we identified the xenobiotic and chemotherapeutic efflux transporter P-glycoprotein (P-gp/MDR1) (18) as well as the copper and cisplatin transporter ATP7B. We could demonstrate that Rab11-FIP1 promoted membrane localisation of P-gp in response to etoposide and cisplatin and enhanced efflux of its substrates (18). Its response to cisplatin is remarkable, as cisplatin is currently not considered a substrate of P-gp. These data suggest a generic response to chemotherapeutics that promotes plasma membrane localisation of Rab11-FIP1 and its cargo (18).

ATP7B is a transmembrane protein which translocates from the Golgi apparatus to the plasma membrane in response to copper overload. Through a copper binding domain, ATP7B binds copper and facilitates efflux of excess copper. However, this binding domain is also responsible for the efflux of cisplatin, which could suggest a role for Rab11-FIP1/mutant p53 in promoting cisplatin efflux through this receptor. The Rab11-FIP1/ATP7B interaction was validated in independent immunoprecipitations in A431 cells exogenously (**Figure 2A**) and endogenously (**Figure 2B**) and both proteins colocalise in cells in the Golgi/vesicular compartment (**Figure 2C**). Similar to P-gp, ATP7B accumulated on the plasma membrane of mutant p53 cells in response to cisplatin, but to a lesser extent in Rab11-FIP1 KO cells (**Figure 2D**). These data suggest that in response to cisplatin, Rab11-FIP1 assists the re-localisation of ATP7B to the plasma membrane. Remarkably, loss of Rab11-FIP1 appeared not to limit the amount of ATP7B expressed on the plasma membrane in response to copper (**Figure 2D**) and Rab11-FIP1 KO cells were not more sensitive to copper exposure (**Figure 2E**).

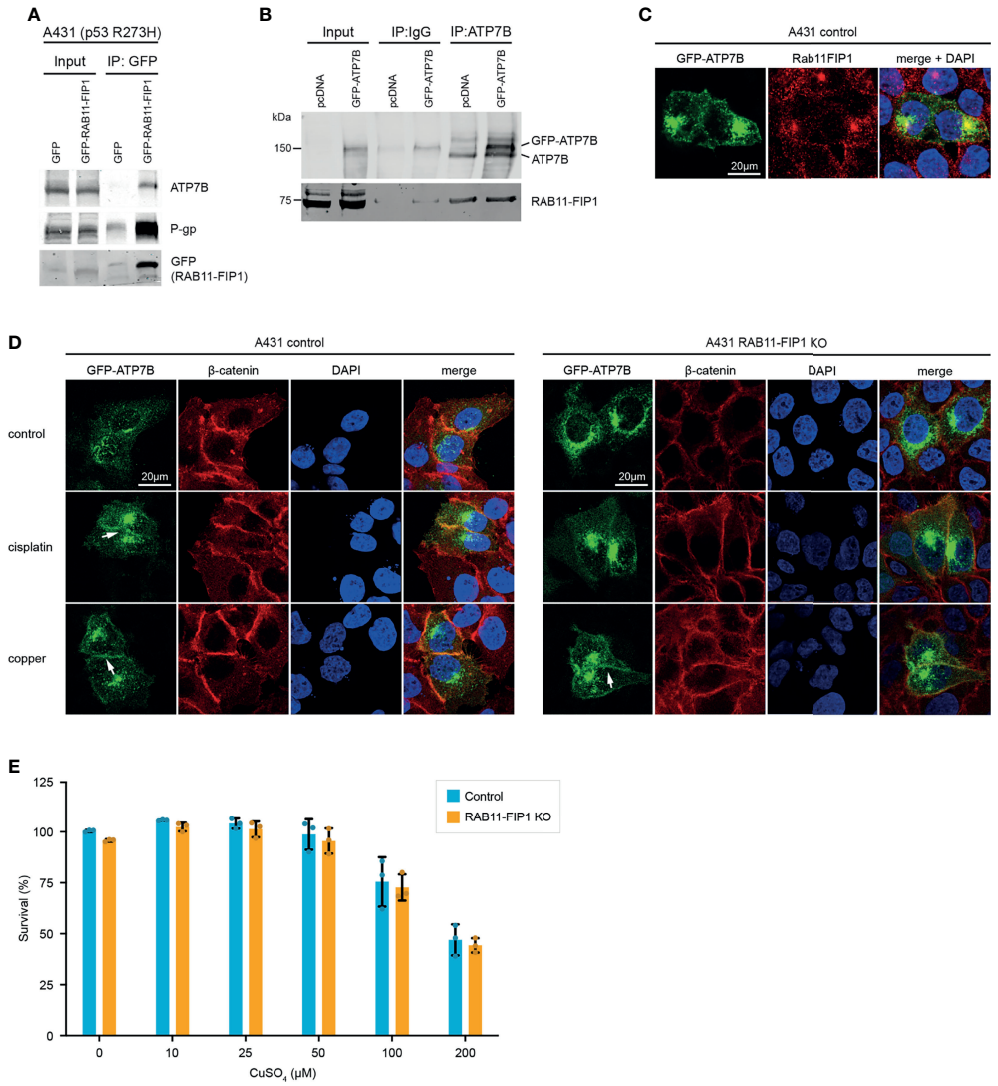

FIGURE 2 | Mutant p53 promotes ATP7B plasma membrane expression in a Rab11-FIP1 dependent manner upon cisplatin stimulation. **(A)** A431 cells expressing mutant p53 (R273H) were transfected to express GFP or GFP-Rab11-FIP1. GFP was immunoprecipitated and co-immunoprecipitation was assessed through western blot, using an ATP7B antibody. **(B)** Co-immunoprecipitation of endogenous ATP7B with Rab11-FIP1 in mutant p53 A431 cells expressing GFP-ATP7B or a GFP control. ATP7B was immunoprecipitated followed by western blot to detect Rab11-FIP1 binding. **(C)** Co-localisation of endogenous Rab11-FIP1 and GFP-ATP7B *(GFP)* was determined using immunofluorescence in A431 cells transfected with GFP-ATP7B **(D)** A431 control or A431 Rab11-FIP1-KO cells transfected with GFP-ATP7B were incubated in cisplatin (3μM) or copper ($CuSO_4$, 100μM) for 2 h and assessed for ATP7B localisation. β-catenin was used as membrane marker and DAPI as nuclear marker. Arrows indicate ATP7B plasma membrane expression. All immunofluorescence experiments were performed in triplicates and assessed in >25 cells per experiment and observer with single plane confocal imaging. Representative images are shown. **(E)** A431 control or A431 Rab11-FIP1-KO cells were incubated in increasing copper concentrations for 72 h and subjected to a resazurin survival assay. Error bars indicate standard deviation of 3 independent experiments (n=3). No statistical significance was observed (unpaired t-test) in copper-treated cells. Materials and methods are provided in **Supplemental Data 1**.

These data could indicate that the type of external stimulus (in this case chemotherapeutics, but not copper) dictates Rab11-FIP1 activity.

Interesting from the aspect of chemoresistance is the role of Rab11 in starvation-induced autophagy. Autophagy is known to play a major role in chemoresistance and Rab11 was shown to be required for autophagosome assembly (50). In response to starvation, Rab11 is relocated from recycling endosomes to autophagosomes (51). Mutant p53 is known to inhibit autophagy, but can also itself be targeted for degradation in response to starvation signals (51). It will be interesting to see if the decrease in mutant p53 expression changes the interaction of Rab11-FIP1 with Rab11 and whether this leads to a redistribution of RTKs, integrins or other receptors.

OTHER MOLECULES REGULATED BY RAB11-FIP1

Rab11-FIP1 has also been shown to interact or co-localise with other proteins and receptors including the TfnR, adiponectin, LRP1B and HIV1 gp160 protein (**Figure 1**), although the functional consequences of these associations remain to be fully elucidated (5, 6, 52–54). Of these, the Rab11-FIP1-dependent regulation of the TfnR, mostly involved in iron uptake, has been most thoroughly studied, but whether Rab11-FIP1 has a role in iron homeostasis is unknown. Interestingly, mutant p53 cells are likely to have elevated iron levels and mutant p53 expression is correlated to elevated transferrin expression (55).

These data could point to a role for Rab11-FIP1 in mediating several distinct downstream signalling pathways downstream of mutant p53 and would make Rab11-FIP1 an interesting target for therapeutic intervention and raises the question of how Rab11-FIP1 is regulated within the cell.

INTRACELLULAR REGULATION OF RAB11-FIP1

In many of the previous settings, it appears that Rab11-FIP1 function can be altered dependent on the stimulus impacted upon cells. In response to EGF, Rab11-FIP1 regulates EGFR membrane expression and in response to etoposide or cisplatin, Rab11-FIP1 promotes expression of P-gp and/or ATP7B to the plasma membrane. Interestingly, Francavilla et al. demonstrated that EGFR recycling can be dependent on the type of signalling (20). When stimulated with TGF-α, EGFR recycling occurred in a Rab11-FIP1 -dependent manner. However, upon EGF incubation, EGFR was recycled in a Rab7-dependent manner, itself dependent on phosphorylation.

The activity of Rab11-FIP1 has been shown regulated by phosphorylation through 2 different kinases so far: Lemur Tyrosine Kinase 3 (LMTK3) and MAP/Microtubule Affinity-Regulating Kinase 2 (MARK2).The LMTK3 kinase promotes S435 phosphorylation of Rab11-FIP1 in response to HGF stimulation (10) and has been studied for its role in breast cancer (56).The MARK2 kinase was shown to promote phosphorylation of S234 in Rab11-FIP1 and promoted polarization of MDCK cells upon a calcium switch. MARK2 is overexpressed in cisplatin-resistant cell lines and expression level of MARK2 correlate with resistance to cisplatin in non-small cell lung cancer (57). This finding suggests that the increase in Rab11-FIP1 activity in mutant p53 cells may be mediated by the MARK2 kinases.

While the activity or expression of these kinases has not directly been linked to mutant p53 expression, it is easy to hypothesise that an increase of Rab11-FIP1 activity could be facilitated through mutant p53-dependent activation of the aforementioned kinases. Several other kinases such as MAP2K3 (58), Aurora kinase A (59) and JNK (60) are regulated by or cooperate with mutant p53. Enhancing the activity of kinases that phosphorylate Rab11-FIP1 would lead to an increased Rab11-FIP1 activity, favouring invasive growth and chemoresistance.

TARGETING THE RAB11-FIP1 SIGNALLING PATHWAY FOR CANCER TREATMENT

With Rab11-FIP1 constituting what appears to be a distribution hub enabling pro-tumorigenic effects through different processes, it could be an interesting drug target in a mutant p53 setting, especially given the fact that mutant p53 targeting therapies are not yet available in the clinic. Several avenues could be explored and can be divided into therapies targeting Rab11-FIP1 itself or any of its downstream effector molecules.

In 2005, Marie et al. reported that Rab11-FIP1 is degraded by calpains in a calcium-dependent manner (61). Increased intracellular Calcium levels (by using the ionophore Ionomycin) reduced Rab11-FIP1 levels. Ionophores such as Ionomycin have been explored as potential anticancer drugs, and might contribute to apoptosis due to increased calcium levels in synergy with chemotherapeutics (62). It will be interesting to explore this strategy in cancers that depend on Rab11-FIP1 and/or mutant p53 expression.

Another way in which Rab11-FIP1 expression levels can be regulated is by the microRNA miR-93. Rab11-FIP1 is a direct target, as demonstrated in cervical cancers in which elevated miR-93 levels coincide with reduced Rab11-FIP1 levels (11). Using cultured cells, Zhang et al. showed that the knockdown of miR-93, allowed for higher Rab11-FIP1 expression, increases apoptosis and reduces proliferation. In that context, miR-93 knockdown seems an interesting approach to reduce tumorigenicity by acting on Rab11-FIP1. However, these findings go against the current "dogma" in the field where elevated Rab11-FIP1 levels and its activation are tumorigenic (11) and might therefore indicate a tissue specific effect, making it pivotal to study Rab11-FIP1's role in different cancers. In other cancers, using miR-93-containing constructs as actual therapeutic could be a strategy now that siRNA therapy has FDA approval to be used in the clinic (63).

Downstream of Rab11-FIP1, EGFR and/or integrin inhibitors have been investigated. Resveratrol and curcumin, which are known to reduce tumour growth, impacted on the expression or activation of these proteins. In oral squamous cell carcinoma development and invasion, Rab11-FIP1 upregulated Zeb1, and subsequently MT1-MMP downstream of β1-integrin/EGFR and β-catenin signalling. Resveratrol inhibited EGFR activation and β1 integrin recycling (21). In cultured SKOV-3 and PA-1 ovarian cancer cells, Rab11-FIP1 promoted invasion by stabilising β1 integrin and activating FAK through EGFR. Interestingly, Curcumin reduced β1 integrin stability, thus reducing EGFR and FAK activation, leading to reduced invasion (64). Most interestingly, resveratrol and curcumin have also been demonstrated to inhibit tumorigenicity of mutant p53 expressing cells. The Silva group has shown in cultured breast cancer cells that Resveratrol inhibits mutant p53

aggregation, but also cell proliferation and migration, thereby reducing tumorigenicity (65). Curcumin was able to re-activate mutant p53 to induce cell death in cultured cells (66, 67), as well as reduce growth of tumour xenografts (66).

Other inhibitors to consider in this respect are direct EGFR inhibitors such as Gefitinib, Erlotinib, Panitumumab and Cetuximab or the integrin inhibitor Cilengitide, which have all been in clinical trials, with some more successful than others. Perhaps, those work best in a setting of mutant p53 and Rab11-FIP1 in which cancer cells are dependent or even addicted to the amplified signalling.

CONCLUSION AND FUTURE PERSPECTIVES

In this perspective, we have seen that mutant p53 can regulate Rab11-FIP1 to modulate a plethora of proteins involved in tumour formation, invasion, metastasis and chemoresistance. Any molecule that could inhibit Rab11-FIP1 could therefore have the potential to stop tumour growth, prevent metastasis and prevent chemoresistance. Some molecules that inhibit downstream pathways of Rab11-FIP1 have shown potential, but strategies that would act on Rab11-FIP1 itself would presumably be more potent. Of interest are the regulation of Rab11-FIP1 by calpains and the degradation upon ionomycin treatment, as well as the potential of targeting Rab11-FIP1 by siRNA. In order to develop a Rab11-FIP1 -based therapy, more research will be needed into the regulation of Rab11-FIP1, its interaction with cargo in different conditions and the cancer-specificity of this response.

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

YvG, conceptualization, visualisation, and writing – original draft. VP and LA, conceptualization and investigation. PM, conceptualization, funding acquisition, methodology, project administration, visualisation, and writing – original draft. All authors contributed to the article and approved the submitted version.

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REVIEW
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Emerging Roles of the Tumor Suppressor p53 in Metabolism

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Metabolism plays critical roles in maintaining the homeostasis of cells. Metabolic abnormalities are often considered as one of the main driving forces for cancer progression, providing energy and substrates of biosynthesis to support neoplastic proliferation effectively. The tumor suppressor p53 is well known for its roles in inducing cell cycle arrest, apoptosis, senescence and ferroptosis. Recently, emerging evidence has shown that p53 is also actively involved in the reprogramming of cellular metabolism. In this review, we focus on recent advances in our understanding of the interplay between p53 and metabolism of glucose, fatty acid as well as amino acid, and discuss how the deregulation of p53 in these processes could lead to cancer.

Keywords: p53, glucose metabolism, lipid metabolism, ferroptosis, amino acid metabolism, iron metabolism

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INTRODUCTION

p53, encoded by the *TP53* gene, is a critical tumor suppressor that is required to prevent the oncogenic transformation of cells. Of note, *TP53* is the most frequently mutated gene in human cancers, and in most cases, *TP53* mutation is associated with poor prognosis (Levine, 2020; Olivier et al., 2010). Mutant p53 (Mutp53) proteins not only lose tumor suppressive functions, but also frequently acquire various gain-of-functions (GOF) that promote tumorigenesis. Under normal conditions, p53 is maintained in an inactive and unstable form through the interaction between p53 and its E3 ligase MDM2 and negative regulator MDMX (Fu et al., 2020). Under various stress conditions, p53 is stabilized and activated by post-translational modifications such as phosphorylation, acetylation, sumoylation, disrupting the interaction between p53 and Mdm2 and Mdmx (Fu, et al., 2020).

As a transcriptional factor, p53 directly activates and suppresses the transcription of hundreds of genes, many of which play key roles in cell cycle, apoptosis, and senescence (Vousden and Prives, 2009). For a long time, the roles of p53 in cell cycle arrest, apoptosis and senescence have been considered the major mechanisms to mediate its tumor suppressive activities (Vousden and Prives, 2009). However, the disruption of p53-dependent cell cycle arrest, apoptosis and senescence is not sufficient to induce cancer (Fu et al., 2020). Instead, various studies in mouse models such as the p53 (3KR/3KR) knock-in mouse model have highlighted its metabolic roles in inhibiting cancer progression (Li et al., 2012).

Reprogramming of cellular metabolism is one of the "hallmarks of cancer", and is considered one of the main driving forces for tumorigenesis (Hanahan and Weinberg, 2011). In order to effectively support neoplastic proliferation, cancer cells increase their uptake of nutrients, especially glucose and amino acids, and adapt themselves to ensure their maximum utilization of the metabolic

intermediates of glycolysis and oxidative phosphorylation for biosynthesis and NADPH production (Pavlova and Thompson, 2016). Numerous reports indicate that p53 is playing extensive and complex roles in regulating various metabolic pathways, and the gain of function mutants of p53 promotes the oncogenic metabolic reprogramming that induces drug resistance and metastasis.

In this review, we focus on recent advances in the research of p53 and its GOF mutants in regulating oncogenic metabolic alterations, aiming to provide insights into the targeted therapy of human cancers with metabolic regulation regiments.

P53 AND GLUCOSE METABOLISM

Numerous studies have shown that p53 plays complex roles in regulating glucose metabolism. Unlike normal cells, tumor cells use glucose mainly through glycolysis rather than oxidative phosphorylation (OXPHOS) to meet their energy and biosynthetic demand even under aerobic conditions, which is known as "Warburg effect" (Warburg et al., 1927). In many cases, p53 performs the tumor suppressive functions to inhibit aerobic glycolysis and promote OXPHOS.

p53 represses the transcription of glucose transporters GLUT1, GLUT3, and GLUT4 to reduce glucose uptake, which is the first rate-limiting event in glycolysis (Kawauchi et al., 2008; Schwartzenberg-Bar-Yoseph et al., 2004). p53 also transcriptionally induces TP53 Induced Glycolysis Regulatory Phosphatase (TIGAR) and inhibits 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3 and PFKFB4), resulting in reduced intracellular levels of fructose-2,6-bisphosphate (F-2,6-BP), which functions as allosteric activator of phosphofructokinase (PFK), the rate-limiting enzyme catalyzing the conversion from F6P to F-1,6-BP (Bensaad et al., 2006; Franklin et al., 2016; Liu et al., 2020; Ros et al., 2017). Moreover, p53 was also reported to inhibit other glycolytic enzymes such as hexokinase 2 (HK2) and phosphoglycerate mutase 1 (PGAM1) (Kondoh et al., 2005; Wang et al., 2014). These findings support the notion that wild-type p53 suppresses glycolysis.

To further tilt the balance from glycolysis to OXPHOS, p53 also promotes cellular OXPHOS by various complementary mechanisms. p53 is able to inhibit the expression of pyruvate dehydrogenase kinase 2 (PDK2), a negative regulator of pyruvate dehydrogenase (PDH) that converts pyruvate to acetyl-CoA, leading to increased OXPHOS (Contractor and Harris, 2012). In addition, p53 induces the expression of Synthesis of Cytochrome C Oxidase 2 (SCO2), thereby promoting the synthesis of cytochrome C oxidase complex that catalyzes the major step of OXPHOS (Matoba et al., 2006). It was also reported that the induction of ferredoxin reductase (FDXR) by p53 promotes electron transfer from NADPH to cytochrome p450 (Liu and Chen, 2002). Moreover, p53 could promote mitochondrial biogenesis, support mitochondrial fission, maintain mitochondrial genome integrity, and ensure the quality control and turnover of mitochondria, thereby guarantees the proper function of mitochondria (Lacroix et al., 2020). Besides, the pentose phosphate pathway (PPP) is also reported to be repressed by p53 through its direct binding to glucose-6-phosphate dehydrogenase (G6PD), which is the first and rate-limiting enzyme of PPP. Consequently, p53 suppresses the production of NADPH as well as precursors for nucleotide biosynthesis (Jiang et al., 2011).

In contrast to the above reviewed canonical functions, the complexity of the roles of p53 in glucose metabolism remains to be elucidated. In this context, p53 could play an oncogenic role by dominantly suppressing OXPHOS. For example, in contrast to many types of human cancers such as lung cancer, wide-type p53 is often retained in hepatocellular carcinomas (HCC), where it induces PUMA expression to disrupt the oligomerization and function of mitochondrial pyruvate carrier (MPC) through direct PUMA-MPC interaction, thereby inhibits the mitochondrial pyruvate uptake and promotes glycolysis of HCC (Kim et al., 2019). These findings underscore the complexity of wild-type p53, indicating that the impact of p53 on glucose metabolism in cancer cells is complex and cell context dependent.

P53, LIPID METABOLISM AND FERROPTOSIS

It has become increasingly clear that cancer cells gain the unique ability to synthesize fatty acids essential for cellular growth and survival (Beloribi-Djefaflia et al., 2016). Another non-canonical function of p53 is the capability to regulate lipid metabolism. p53 is thought to promote catabolism of fatty acids while simultaneously inhibit fatty acid synthesis. In addition to its inhibition of Glucose-6-phosphate dehydrogenase (G6PD) and pentose phosphate pathway (PPP) that is important for DNA synthesis and lipid synthesis (Jiang, et al., 2011), p53 can transcriptionally upregulate aromatase that is involved in lipid metabolism (Wang et al., 2013). Increased lipid accumulation in the livers of $p53^{-/-}$ mice is mitigated by the transgenic expression of aromatase, indicating important roles of p53-aromatase pathway in lipid metabolism (Wang, et al., 2013).

While wild-type p53 can suppress lipid synthesis by regulating the activities or levels of downstream effectors/targets such as G6PD and aromatase (Jiang, et al., 2011; Wang, et al., 2013), numerous reports have demonstrated that mutant p53 can promote lipid synthesis by altering the activities of various transcription factors or signaling molecules such as p63, p73, Nrf2, and AMP-activated protein kinase (AMPK), which are involved in lipid metabolism (Do et al., 2012; Walerych et al., 2016; Xu et al., 2011). Several studies have shown that the upregulation of enzymes involved in the synthesis of fatty acids and cholesterol (mevalonate pathway) is required for tumor progression (Bathaie et al., 2017; Kuhajda et al., 1994; Ribas et al., 2016; Roongta et al., 2011; Zhan et al., 2008). The presence of p53 mutations correlates with high levels of enzymes involved in the mevalonate pathway in human breast cancer tissues (Freed-Pastor et al., 2012). Another study shows that ectopic expression of p53 mutants ($p53^{R175H}$ and $p53^{P151S}$) inhibits AMPK activity and subsequently reduces phosphorylation of Acetyl-CoA carboxylase (ACC) under

glucose and serum starvation in a p53-null head and neck squamous cell carcinoma (HNSCC) cell line UMSCC1 (Zhou et al., 2014).

Ferroptosis is a new form of programmed cell death characterized by the accumulation of iron-dependent lethal lipid peroxides (Dixon et al., 2012). p53 plays an important role in modulating ferroptotic responses by regulating the expression of its metabolic targets (Jiang et al., 2015). For example, recent studies have shown that ALOX12 is critical for p53-mediated ferroptosis (Chu et al., 2019). In addition, p53 induces ferroptosis partly through transcriptional activation of Glutaminase 2 (Jennis et al., 2016) and SAT1 (a polyamine catabolic enzyme) (Ou et al., 2016), and transcriptionally represses *SLC7A11* (Jiang, et al., 2015). In addition, suppressor of cytokine signal transduction protein 1 (SOCS1) is required for p53-mediated expression of p53 target genes involved in ferroptosis. In this context, SOCS1 can reduce the expression of SLC7A11 to sensitize cells to ferroptosis (Saint-Germain et al., 2017). However, p53 behaves differently in a context dependent manner. While the basal p53 promotes ferroptosis, stress-induced p53 can inhibit ferroptosis (Tarangelo et al., 2018; Xie et al., 2017). For example, p53 inhibits ferroptosis by inhibiting dipeptidyl-peptidase-4 (DPP4) activity in human colorectal cancer cell lines (Xie, et al., 2017). Therefore, further studies would be needed to clarify the complex roles of p53 in ferroptosis.

P53 AND IRON METABOLISM

In addition to ferroptosis, p53 also modulates iron homeostasis. p53 expression is decreased upon the exposure to excessive levels of iron through heme-p53 interaction (Shen et al., 2014). Under iron-deprived conditions, HIF1α is activated to increase p53 protein stability and protein levels (An et al., 1998; Peyssonnaux et al., 2008; Peyssonnaux et al., 2007). In contrast, p53 is also found to be downregulated upon iron depletion via MDM2 (Dongiovanni et al., 2010). Therefore, the regulatory mechanisms of p53 by the iron concentration appear to be context dependent.

p53 can control the intracellular iron pool by modulating the expression of iron sensors. For example, p53 directly activates the expression of hepcidin, an iron-regulating hormone (Weizer-Stern et al., 2007). Another study suggests that p53 induces the expression of iron-sulfur cluster assembly proteins (ISCU) and protects cells from iron overload (Funauchi et al., 2015). p53 has been reported to modulate mitochondrial proteins that are involved in iron metabolism. For example, p53 mediates the expression of its target ferredoxin reductase (FDXR), and subsequently, modulates mitochondrial iron homeostasis through iron sulfur clusters (ISC) or heme synthesis (Sheftel et al., 2010).

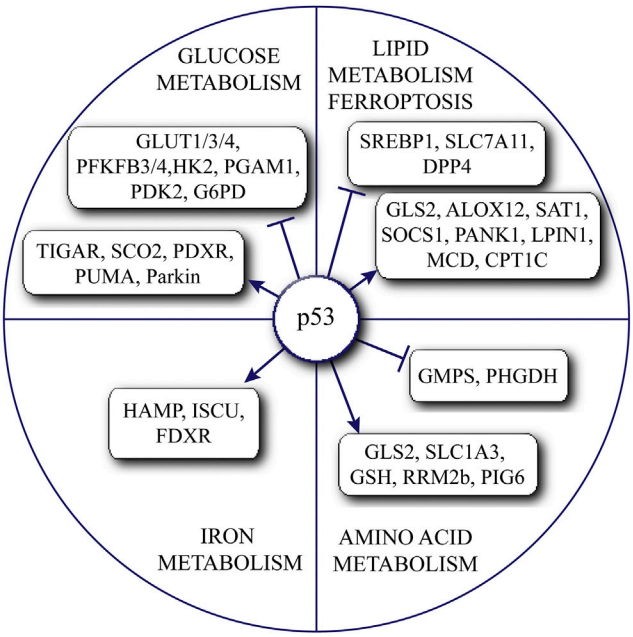

FIGURE 1 | Summary of the complex roles of p53 in regulating various metabolic pathways.

P53 AND AMINO ACID METABOLISM

Amino acid metabolism has extensive effects on tumors, and it has been revealed that p53 functions to protect cells from metabolic stress and promote cellular survival. Cancer cells rely on glutamine for cellular proliferation after glucose depletion through a process named glutaminolysis, by which glutamine is converted to the intermediates of the TCA cycle (Pavlova and Thompson, 2016). p53 activates the expression of Glutaminase 2 (GLS2), a key enzyme in glutamine-based cellular energy production under glucose-deprivation conditions to support cancer cell growth. Glutamate also limits intracellular and extracellular oxidative stress to promote cell survival (Suzuki et al., 2010).

Under the conditions when both glucose and glutamine become limited, aspartate metabolism becomes very important for cellular energy production. p53 is reported to transactivate Solute Carrier Family 1 Member 3 (SLC1A3), an aspartate/glutamate transporter, under glutamine starvation conditions (Tajan et al., 2018). p53 can also promote cellular survival by the induction of high affinity amino acid transporter Solute Carrier Family 1 Member 3 (SLC1A3) (Tajan, et al., 2018). Another important player in tumor cell survival and proliferation is serine. p53 promotes serine synthesis by glutathionine (GSH) synthesis, eventually leading to overall cell survival (Maddocks et al., 2013). In summary, p53 promotes cellular survival by promoting energy production from amino acids under the condition of glucose deprivation.

CONCLUDING REMARKS

Tumor suppressor gene p53 is not only essential in cell cycle arrest or apoptosis, but also participates in various physiological functions. Here we take a closer look at the complexity of p53 function in regulating cellular metabolism. These findings together suggest that p53 could regulate various aspects of cellular metabolism via regulating different target gene expression or protein-protein interactions in a cellular and environmental context dependent manner (**Figure 1**). The roles of wild-type p53 in tumor metabolism are complex, and sometimes, could conflict with its status as a tumor suppressor. For example, some roles of p53 in suppressing OXPHOS and inducing amino acid based energy production can promote cancer cell survival and proliferation (Kim, et al., 2019; Suzuki, et al., 2010, Tajan et al., 201). While the full-length p53 mutants are found to be overexpressed in more than half of human cancers and apparently gain new oncogenic properties (Zhu et al., 2020), many questions remain unanswered for their roles in cellular metabolism. Further advancements in single-cell analysis and multi-omics analyses will provide more in-depth understanding of p53-related regulatory mechanisms.

Considering the unusual reliance of cancer cells on glycolysis, targeting tumor metabolic reprogramming has become a promising strategy for cancer treatment. In addition, the increased glycolysis contributes to higher levels of the acidic intermediates such as lactate and acidic tumor microenviroment, directly or indirectly suppress tumor immunity. Therefore, the activation of the roles of p53 in suppressing the metabolic reprogramming of cancer cells could become effective targeted therapy for human cancers. However, the development of such strategy requires attention to the complex and sometimes conflicting roles of p53 in cancer cells. The comprehensive understanding of various p53 regulated pathways will enable the precise activation of the p53-dependent pathways in suppressing tumor metabolism.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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p53 Modulation of Autophagy Signaling in Cancer Therapies: Perspectives Mechanism and Therapeutic Targets

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The key tumor suppressor protein p53, additionally known as p53, represents an attractive target for the development and management of anti-cancer therapies. p53 has been implicated as a tumor suppressor protein that has multiple aspects of biological function comprising energy metabolism, cell cycle arrest, apoptosis, growth and differentiation, senescence, oxidative stress, angiogenesis, and cancer biology. Autophagy, a cellular self-defense system, is an evolutionarily conserved catabolic process involved in various physiological processes that maintain cellular homeostasis. Numerous studies have found that p53 modulates autophagy, although the relationship between p53 and autophagy is relatively complex and not well understood. Recently, several experimental studies have been reported that p53 can act both an inhibitor and an activator of autophagy which depend on its cellular localization as well as its mode of action. Emerging evidences have been suggested that the dual role of p53 which suppresses and stimulates autophagy in various cencer cells. It has been found that p53 suppression and activation are important to modulate autophagy for tumor promotion and cancer treatment. On the other hand, activation of autophagy by p53 has been recommended as a protective function of p53. Therefore, elucidation of the new functions of p53 and autophagy could contribute to the development of novel therapeutic approaches in cancer biology. However, the underlying molecular mechanisms of p53 and autophagy shows reciprocal functional interaction that is a major importance for cancer treatment and manegement. Additionally, several synthetic drugs and phytochemicals have been targeted to modulate p53 signaling via regulation of autophagy pathway in cancer cells. This review emphasizes the current perspectives and the role of p53 as the main regulator of autophagy-mediated novel therapeutic approaches against cancer treatment and managements.

Keywords: p53, autophagy, apoptosis, tumor suppressor, synthetic drug, phytochemical

INTRODUCTION

Autophagy, a self-degradative intracellular process, is an essential mechanism of the cell that facilitates renewal or removal of cellular molecules, thereby balancing the cell's energy consumption and maintaining homeostasis (Rahman and Rhim, 2017; Rahman et al., 2020a). However, autophagy deregulation is now considered to be one of the most characteristic features for tumor progression (White, 2015). It has recently been revealed that autophagy suppression and a combination of chemotherapeutic treatment have been approached as a potential treatment for cancer (Perez-Hernandez et al., 2019), although this depends on the context and type of cancer. To date, numerous tumor suppressor oncogenes and proteins have emerged as eminent autophagy regulators whose mutation or depletion regulates autophagy as well as tumorigenesis. Evidences have been suggested that p53 which belonging to the tumor suppressor genes may act as an inhibitor or activator of autophagy depending on their mode of action and subcellular localization (Lacroix et al., 2020). Morevoer, physiological role of autophagy in cancer offers a highest possible target for future cancer therapy and is, hence, presently intensively investigated. Therefore, understanding p53 regulation and its role in individual cellular contexts with a suitable approach of autophagy-mediated regulation in cancer is crucial for drugs development that might be targeted autophagy in a specific diseases model.

Tumor suppressor p53 has been implicated in a wide variety of cellular processes, including genomic stability, cell-cycle arrest, DNA repair, apoptosis, cellular senescence, and autophagy (Aubrey et al., 2018; Mrakovcic and Frohlich, 2018). Generally, p53 binds to DNA in the nucleus which regulates transcription of target genes to activate apoptosis (Tang et al., 2021b). Nevertheless, human p53 mutation has been encouraged tumor progression, chemoresistance, and apoptosis (Alvarado-Ortiz et al., 2021). Additionally, p53 inactivation is effectively used as a therapeutic target of a promising approach to trigger anti-cancer therapy (Zawacka-Pankau and Selivanova, 2015). Thus, p53 has a dual role as a positive or negative regulator of autophagy in cancer (Liu and Gu, 2021). Under normal cellular conditions, p53 has been recognized as an autophagy inhibitor, while in response to stress or starvation, p53 might be translocated into the nucleus which endorsed autophagy via transactivation with its target genes (Mrakovcic and Frohlich, 2018; Fang et al., 2021). p53 functions have been modulated via several post-translational modifications as well as different interacting proteins (Soussi, 2000). Among them, 14-3-3 family proteins play an important function in p53 regulation in response to DNA damage (Falcicchio et al., 2020). However, reasons for this difference in wild-type and mutant p53 activities have been triggered apoptosis and cell cycle arrest remain unclear (Parrales and Iwakuma, 2015). Particularly, experimental studies have been confirmed that mutant with gain-of-function variant of p53 in tumors cells are characterized via a higher genomic instability in response to reduce chemotherapeutic which has usually poor prognosis for patients (Liu et al., 2012). In this review, the molecular mechanisms and regulation of autophagy in cancer would be discussed regarding modulation of p53. Additionally, recent progress of autophagy signaling in tumor microenvironment in addition to its targeting for possible cancer therapeutics developments from the pre-clinical trials anong with the challenges in developing autophagy-based cancer therapy (Mukhopadhyay et al., 2021). Therefore, current approaches triggering p53-mediated autophagy regulation in cancer treatment are highlighted and summarized in cancer cells to conventional treatments which are able to overcome chemoresistance in cancer.

METHODS

Literature-based online databases, Google Scholar, Web of Science, PubMed, Google, and Scopus were accessed to collect information on the published articles that reported molecular mechanism of p53 and autophagy modulation in cancer prevention. Several keywords were used in the search, such as p53, autophagy, cancer, phytochemicals, natural compounds, solid tumors, and lymphomas perspectives role of p53 and autophagy in cancer therapy. Figures were created with the Adobe Illustrator software.

BIOLOGICAL FUNCTION OF P53 SIGNALING IN CANCER

The p53 is a central transcription factor that has the capacity to induce diverse cellular responses likely DNA damage repair, cell cycle arrest, apoptosis, and senescence followed by various stress signals (**Figure 1**) (Subburayan et al., 2018; Mijit et al., 2020). The master biological function of p53 is to ensure the safety of the DNA uprightness of the cell (Munroe et al., 2020). Along with this, p53 protein operates some additional acts in cellular aging, cell differentiation, and development (Jain and Barton, 2018). The p53 antitumor function is broadly governed by dual approaches; it can promote repair of the DNA damage or promote apoptosis or autophagy to completely remove the irreplaceable damaged materials or cells (Crighton et al., 2006; Janicke et al., 2008). In fact, p53 is a transcription factor of the nucleus which governs the diverse array of cellular processes and escorts transcription of a broad group of target genes of it. At the initial phase of DNA damage, p53 activates and induce cell-cycle arrest of G1-phage which is attributed to repair the DNA damage by promoting the transcription of p21WAF1, GADD45, and p53R2 (He et al., 2020). Following the DNA repair, cells can start come back into the regular cell cycle procedure resulting in p53 itself regulate nuclear integrity to prohibit tumor induction or occurrence (Williams and Schumacher, 2016; Cafaro et al., 2020). On the other hand, p53 is able to apply its pro-apoptotic activities

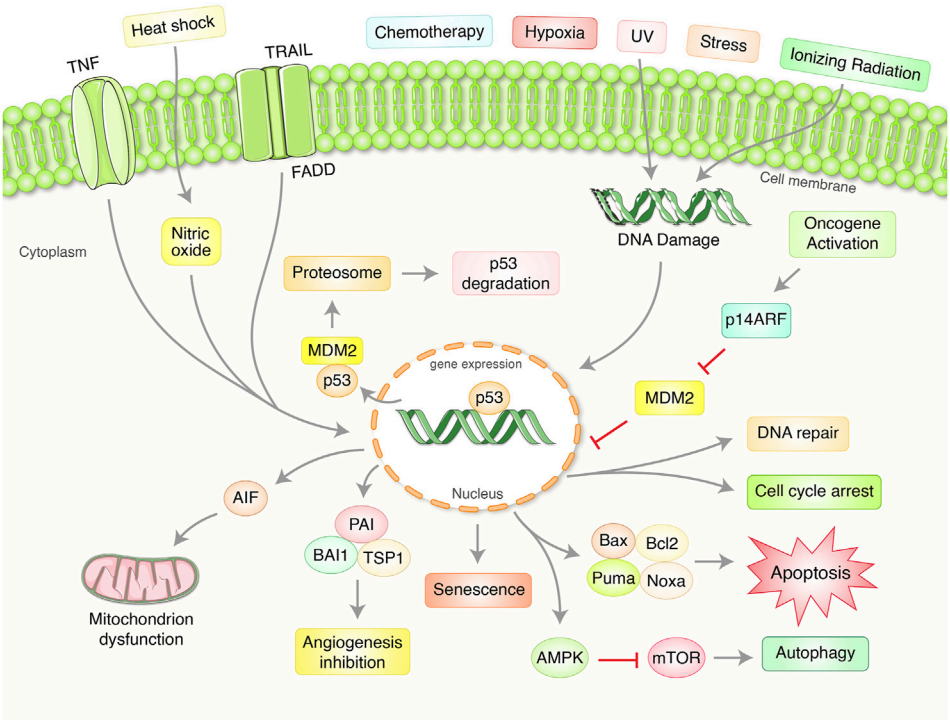

FIGURE 1 | Importance and regulation of tumor suppressor p53 pathway in the regulation of cancer. p53 protein plays an essential role in coordinating with a complex signaling network which regulate aberrant cell proliferation and growth. Normally, p53 has preserved at low steady-state levels with crucial regulation of two proteins, murine double minute 2 (MDM2) as well as MDMX. MDM2 mediates an attachment of ubiquitin (Ub)-mediated proteasomal degradation. Exposure to ultraviolet (UV) light and ionizing radiation activate several kinases and damaging stressors. Oncogenes overexpression has been found to stimulate the production of alternative reading frame (ARF), p14ARF in human and p19ARF in mouse, which binds to MDM2 as well as stabilizes p53. Activation of p53 protein has been targeted to transactivate numerous gene expressions depending on the stressors and the cell type which significantly control either DNA repair, cell cycle arrest, senescence, apoptosis, mitochondrion regulation, autophagy, and angiogenesis.

through the removal of the damaged cells (Ingaramo et al., 2018). In this cellular process, p53 is responsible for the transactivation of a wide range of pro-apoptotic target genes that encodes Bax, Bak, Puma, and Noxa proteins belong to BH-3 only protein and playing a role to promote apoptosis in a cell (Moll et al., 2006; Labi et al., 2008) (**Figure 1**). In this manner, p53 can protect from tumorigenesis or cancer initiation by regulating this complex process.

Role of Mutant p53 Contributes to Autophagy Regulation in Cancer

It has been found that mutant p53 proteins are involved in different autophagic pathways vai degrading and targeting to explore the potential approaches in cancer through autophagy (Shim et al., 2021). p53 mutant has designated as a gain-of-oncogenic function(s) (GOFs) which improved cell migration, proliferation, as well as invasion with anti-apoptotic functions which dynamically contribute to numerous phases of tumor progression in cancer (Dittmer et al., 1993; Oren and Rotter, 2010). The changes beyond cancerous are subjected to deliberate discriminating benefits such as facilitating angiogenesis, continuous growth avoiding growth signal, insensitivity to cancer drugs, promotes adequate metabolism, escape from apoptosis with the self-sufficiency of stress signal and ultimately promoting metastasize and invasion (Chatterjee and Viswanathan, 2021; Hernandez Borrero and El-Deiry, 2021). Furthermore, growing evidences from *in vitro* and *in vivo* have signified that the oncogenic activities of p53 mutant variants have heterogeneous which can vary with tissue type in addition to genetic background of the cells (Eriksson et al., 2017). Almost 50% of the p53 gene is mutated in cancer cells, which underlying its normal role in cancer suppression, favors interchange or inactivate the gene which gains a new function that cooperates to sustain the abnormal growth of cancer (Boutelle and Attardi, 2021). Additionally, mutant p53 proteins have been found to exert on autophagy while other mutant p53 activities might affect diverse aspects of cancer biology. It was found that ectopically overexpressing 22 different p53 mutant variants control autophagy in p53 null colon cancer cells (Morselli et al., 2008). $p53^{R175H}$ or $p53^{R273H}$ mutants suppresses autophagic vesicles formation and lysosomes fusion via the transcriptional suppression of p53 key downstream responsive autophagy related protein such as DRAM1, BECN1, ATG12, SESN1/2, P-AMPK, and TSC2 (Cordani et al., 2016). Furthermore, protein-protein interactions with other transcription factors as a GOF and some cancer-associated p53 mutants have been shown the capability to block

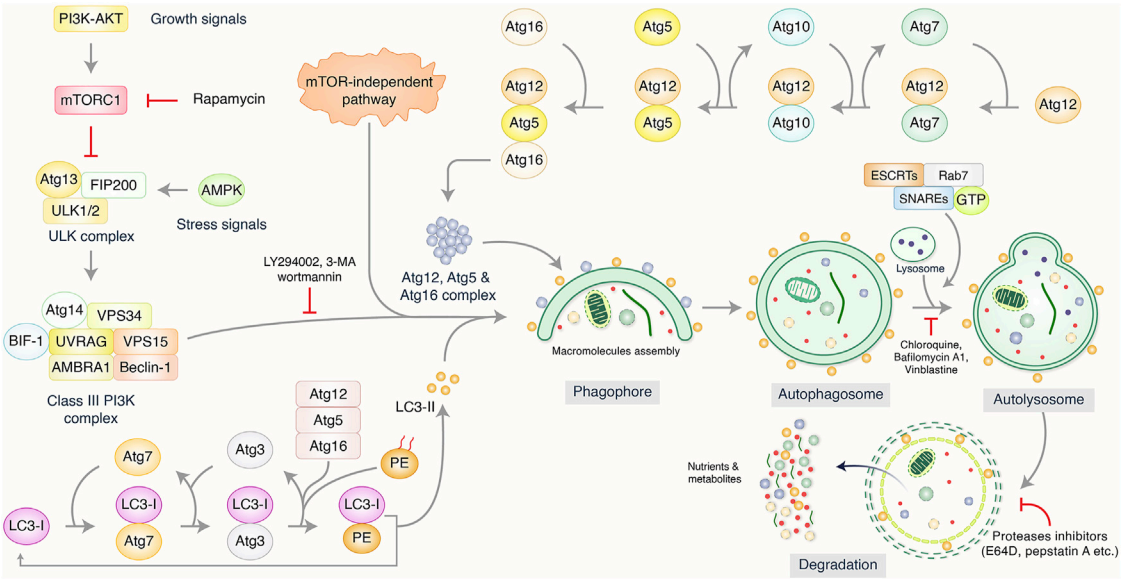

FIGURE 2 | Biological function and molecular mechanism of autophagy pathway. Autophagy has been initiated by the formation of pre-autophagosome structure via the action of several proteins. PI3K-AKT and mammalian target of rapamycin (mTOR) have been influenced to initiate pre-autophagosome assembly via association of ULK1/VPS34/Beclin-1 complex. Additionally, Atg5/Atg12/Atg16 and Atg12/Atg5/LC3 complexes are involved to create phagophore nucleation and macromolecules accumulation which has been elongated as well as bind to autophagosome formation. Lysosome binds mature autophagosome by the help of ESCRT/SNARE/Rab7 protein complex, resulting in autolysosome formation. Finally, autolysosomes have been abolished by acid hydrolases resulting in the release of recycling metabolites as well as nutrients.

autophagy indirectly via triggering numerous growth factor receptors as EGFR, TGFBR, and IGFR which contributing to sustain PI3K/Akt/mTOR signaling and subsequently suppress autophagy in cancer (Aschauer and Muller, 2016). Therefore, targeting of p53 mutant proteins by autophagy inhibition and activation might offer a promising future therapeutic opportunity and is thus presently investigated intensively to modulate autophagy in cancer therapies.

BIOLOGICAL FUNCTION OF AUTOPHAGY IN CANCER

Autophagy has been categorized as an intracellular self-degradation mechanism through dysfunctional cytoplasmic organelles and aggregated misfolded proteins are terminated via fusion with lysosomes and double-membrane autophagosomes to maintain cellular homeostasis (Krishnan et al., 2020; Miller and Thorburn, 2021). Usually, autophagy process, mainly macroautophagy, has been initiated via the isolation of pre-autophagosome structures called phagophore assembly sites (PAS) (Hurley and Young, 2017). PI3K related to the endoplasmic reticulum (ER) have a vital role to initiate PAS formation (Kotani et al., 2018). Unc-51 like autophagy activating kinase-1 (ULK1), mammalian target of rapamycin (mTOR), and AMP-activated protein kinase (AMPK) facilitate phagophore formation during induction of autophagy (Alers et al., 2012; Rahman et al., 2021a). Nevertheless, VPS34/UVRAG/Beclin-1/AMBRA1 helps in the phagophore formation (Velazquez and Jackson, 2018), followed by membrane elongation and autophagosome formation (Rubinsztein et al., 2012). Lysosome binds to mature autophagosome by the association of ESCRT/SNARE/Rab7 protein complex, resulting in the formation of autolysosomes (Kardideh et al., 2019; Rahman et al., 2021c). Finally, autolysosomes that contain misfolded/aggregated proteins have been degraded via acid hydrolases and provide recycling metabolites and nutrients for maintaining intracellular homeostasis (**Figure 2**). It has been found that cancer cell fate regulations and development depended on the autophagy process (Wei and Huang, 2019).

Additionally, well-known cellular autophagy mechanism contributing to carcinogenesis is chaperone-mediated autophagy (CMA) which signify lysosomal-mediated degradation process to facilitate cell survival (Chava et al., 2017). It has been found that during serum starvation, CMA and macroautophagy are triggered consecutively signifying that these two paths are not entirely independent while deficiency or blockage one of this pathway may lead to activate other (Kaushik et al., 2008; Cuervo and Wong, 2014). However, CMA has been found to degrade mutant p53 in a lysosome-dependent fashion in cancer cells under nonproliferating conditions (Vakifahmetoglu-Norberg et al., 2016). Later, chaperone-assisted selective autophagy (CASA) was found in skeletal muscle cells which coordinates protein synthesis and degradation and act as an important physiological stimulus crucial for cellular development, respiratory, maintain urogenital systems, and homeostasis of locomotory (Ulbricht et al., 2013). Moreover, CASA machinery ensures proteostasis in addition to regulate

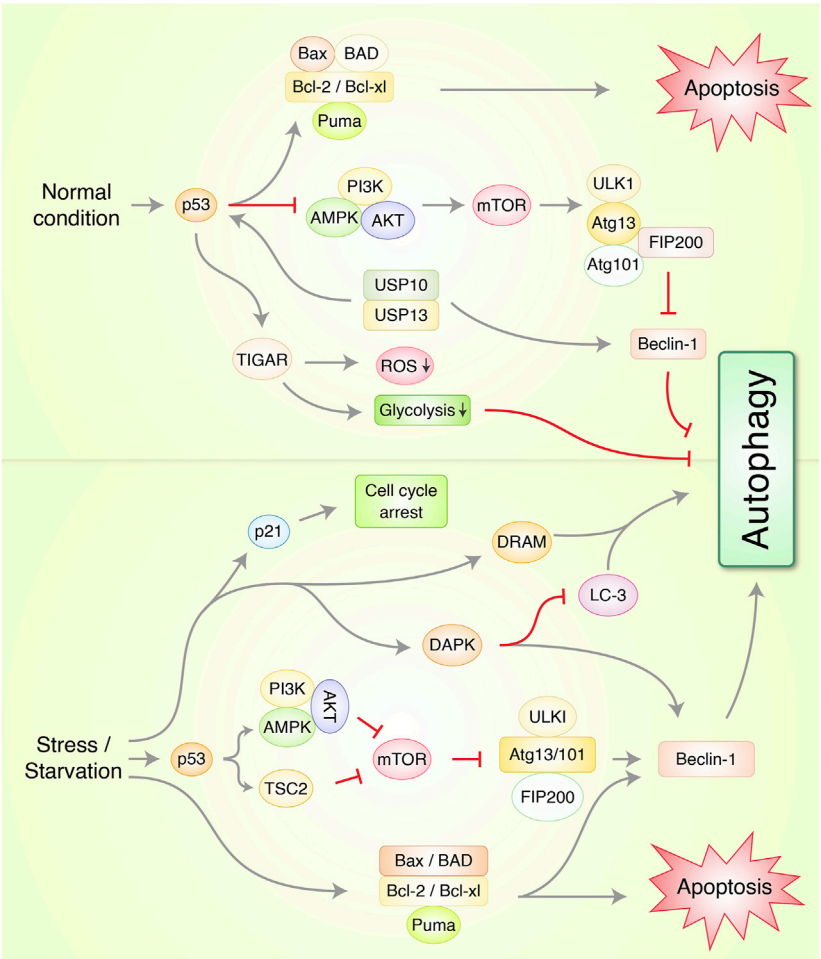

FIGURE 3 | Molecular mechanism of autophagy and apoptosis via p53 regulation in cancer under normal and stress conditions. In normal condition, p53 protein prevents autophagy-mediated cell death via induction of Beclin-1 degradation through ubiquitin-specific peptidases USP10/USP13 and AMPK/mTOR/ULK1 complex activation. TP53-induced glycolysis and apoptosis regulator (TIGAR) prevents autophagy through suppression of reactive oxygen species (ROS) and glycolysis formation. Under stress/starvation condition, p53 activates AMPK and tuberous sclerosis complex 2 (TSC2) which suppresses mTOR and ULK1/FIP200 complex which finally stimulates autophagy. Cyclin-dependent kinase inhibitor 1, p21, activates and arrests cell cycle via p53-mediated upregulation. Additionally, death-associated protein kinase (DAPK), and damage-regulated autophagy modulator (DRAM), autophagy-related protein Beclin-1 upregulation initiates autophagy. Bcl-2 family, Bcl-2, Bcl-xl, Bax, Bad, and PUMA activates apoptosis.

essential cellular developments such as proliferation, migration, and adhesion which comprises the molecular chaperones HscA8/Hsp70 as well as HspB8/Hsp22 alone with the co-chaperones Bag3 and STUB1/CHIP (Liu et al., 2013). Importantly, it is found that CASA is essential for muscle maintenance (Arndt et al., 2010). Therefore, HscA8/Hsp70 and HspB8/Hsp22 compex and CASA play an significant function in protein quality control of cancer cells.

Accumulating evidence indicated that autophagy could decide whether cancer cells are promoted or suppressed in certain conditions (Rahman et al., 2020a). In that case, mTOR has an essential function either cellular function becomes an oncogenic activating or protective via inactivation or induction of autophagy pathway (Uddin M. S. et al., 2020; Rahman et al., 2021b). In addition, chemotherapeutic drugs were shown to suppress tumor cells by autophagic modulation (Rahman et al., 2020b). Also, autophagy inhibition has been regulated in cancer progression which decides whether autophagy influences cell death or cell survival function (Jung et al., 2020). Furthermore, epigenetic and genetic function might be alternated the Atgs gene expression which has a greater impact on cancer cell survival. Thus, autophagy modulation of cancer cells has been found to examine the distribution of tumor microenvironment progression which contributes to the potential management and prevention of cancer (Rahman et al., 2020b). Therefore, p53 may react to different kinds of stress as well as damage employed on the cell which comprise endogenous- or environmentally-stressed genotoxicity, oxidative stress, and oncogene activation in order to protect cell damage as well as maintain cellular integrity in cancer (Liu and Gu, 2021). Nevertheless, how posttranslational modifications of p53 postulate its selectivity for each of these transcriptional targets

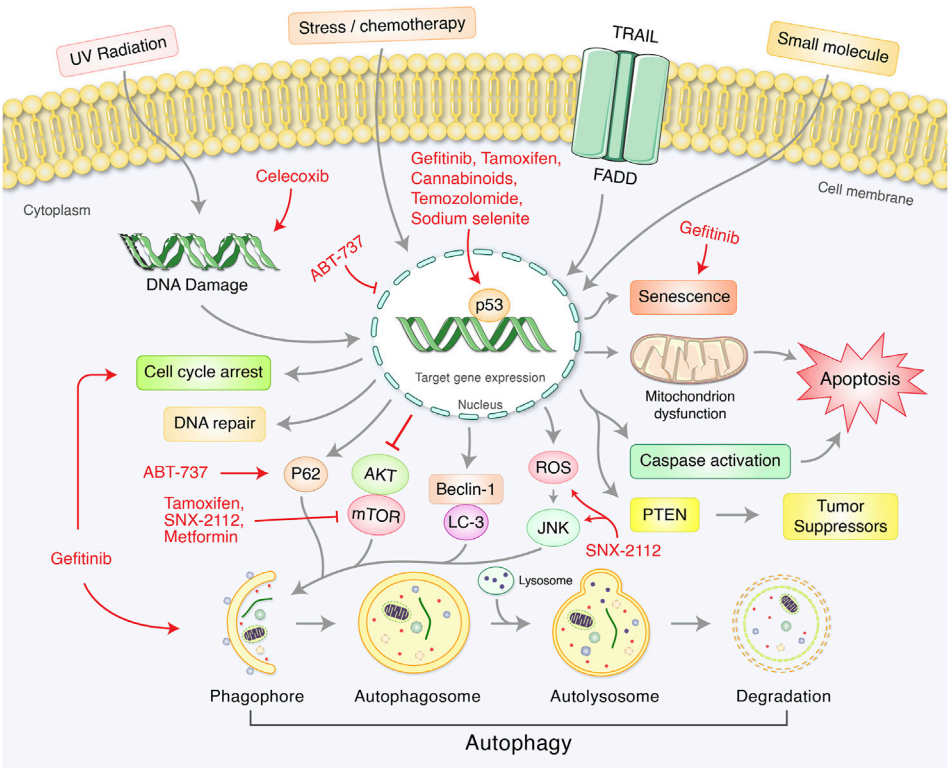

FIGURE 4 | Synthetic drugs targets for p53-mediated autophagy modulation in cancer therapy.

as well as the particular cellular function which induce autophagy in cancer is still unclear.

P53 SIGNALING TARGETS AS A CANCER THERAPY VIA MODULATION OF AUTOPHAGY

Cell death regulation is a complicated process of maintaining cellular homeostasis by preventing oncogenic growth and recycling damaged cell debris (Rangel et al., 2021). Dysregulation of autophagic cell death occurs frequently in a variety of malignancies and poses a barrier to current therapy development Rahman et al., 2020. Autophagy plays a critical role in both tumor promotion and suppression. Autophagosomes engulf and digest cell organelles and proteins, which are then recycled to restore homeostasis and cellular metabolism (Duffy et al., 2015). In recent years, it has been proposed that the suppression of autophagy in combination with chemotherapy could be used as an innovative way to treat cancer (**Figure 3**). Interference with the autophagic machinery, on the other hand, can promote or disrupt carcinogenesis, depending on the type of cancer and their environment. It is, therefore, critical to uncover the primary signaling mechanisms that control carcinogenesis and regulate autophagy (Mrakovcic and Frohlich, 2018). Recently, it has been found that autophagy enhanced the stemness of lung CSCs via degrading ubiquitinated p53, therefore relieving cytosolic p53 inhibition of autophagy through generating stable human lung CSC cell lines of wild-type TP53 (A549) where TP53 has been deleted (H1229) (Wang J. et al., 2021).

The research to date has found several tumor suppressor proteins and oncogenes to be essential regulators of autophagy. The loss or mutation of these proteins contributes to tumor formation. In addition to being one of these tumor suppressors, the mammalian cell "janitor" p53 may be one of the most frequently mutated genes in human tumors. Most human cancers exhibit p53 mutation, which is found in approximately half of all tumors (Soussi and Wiman, 2007; Shi Y. et al., 2020). P53 activation is dependent on various stressors, such as DNA damaging agents, oncogenes, and hypoxia, as well as others, and leads to changes in cell cycling, apoptosis, senescence, metabolism, differentiation, as well as angiogenesis inhibition and autophagy control (Giaccia and Kastan, 1998; Levine and Abrams, 2008). From the results of recent experimental research, it has been ascertained that p53 has both an activator and an inhibitor function with regard to autophagy, depending on its cellular localization and the way of operations (Mrakovcic and Frohlich, 2018). p53 may play a pro-autophagic role in the nucleus, both in a transcription-dependent and independent manner. In the cytoplasm, on the other hand, p53 is known to suppress the induction of autophagy (Maiuri et al., 2010). Recently, it has been highlighted that interplay between pro-inflammatory/pro-oncogenic and pro-inflammatory cytokines

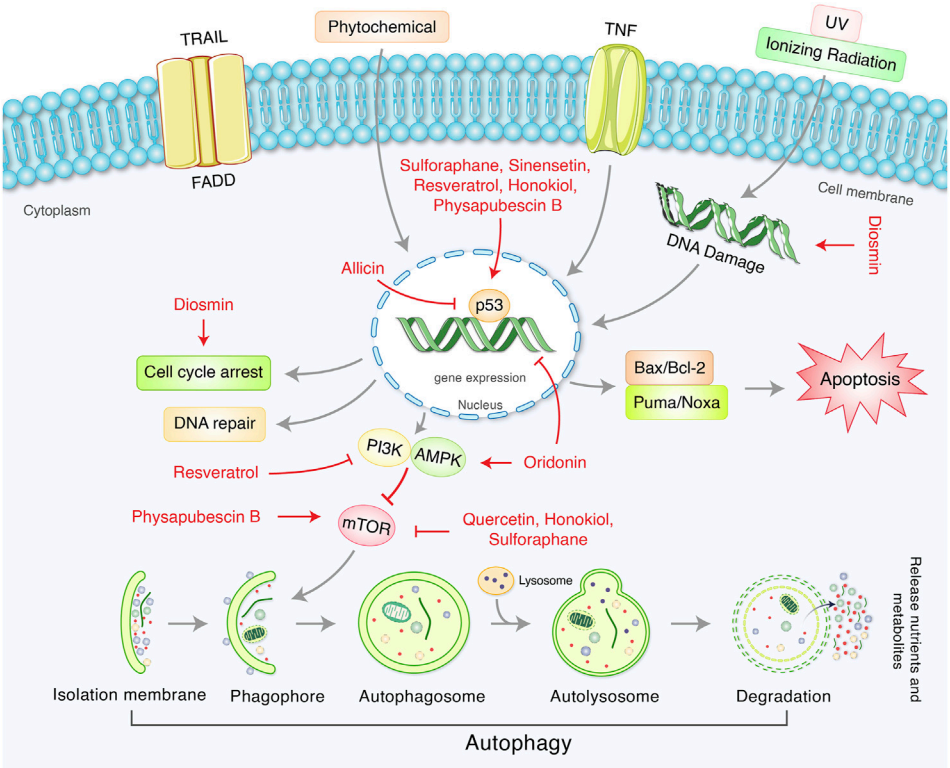

FIGURE 5 | Therapeutic actions of phytochemicals and their targeted signaling system in p53-mediated autophagy regulation in cancer.

pathways regulated via UPR signaling as well as autophagy which affects the stability of p53 that is able to control UPR signaling, cytokine release, and autophagy to preserve its own stability in additional to promote tumorigenesis against cancers carrying mutp53 (D'Orazi et al., 2021).

There are many different cell stressors that might activate p53 (Punja et al., 2021). It is possible that activated p53 may downregulate the autophagy negative regulator, mTOR, through transcriptional regulation of Sestrin1 and Sestrin2, which activate AMPK, which then phosphorylates tuberous sclerosis 2 protein (TSC2) (Budanov and Karin, 2008; Maiuri et al., 2009). In addition to AMPKβ1 and AMPKβ2, p53 can transactivate other AMPKβ subunits including TSC2, PTEN, and IGF-BP3. All of these AMPKβ subunits can be upregulated in response to a stress signal, and upon this elevation, the p53-dependent negative regulation of the mTOR pathways takes place (Feng et al., 2007; Eby et al., 2010). All of these AMPK subunits are capable of being upregulated in response to a stress signal, and this upregulation results in the p53-dependent negative regulation of the mTOR pathways (Jazvinscak Jembrek et al., 2021). A number of targeted genes are activated or inhibited by p53, suggesting that autophagy and cancer prevention are achieved through p53 actions (e.g., activating AMPK and inhibiting mTOR). DRAM (damage-regulated autophagy modulator), a p53 target gene encoding a lysosomal protein that induces macroautophagy, is another mechanism by which p53 promotes the activation of the autophagic pathway. In addition, AEN/ISG20L1 was found to modulate autophagy in response to genotoxic stress by interacting with members of the p53 family (Eby et al., 2010). The three p53 family members (p53, p63, and p73) can regulate transcription of AEN, and downregulation of AEN expression results in decreased levels of autophagic vacuoles and LC3-II, which indicates genotoxic stress. In addition to positive regulators of autophagy, several other pro-apoptotic genes such as PUMA (p53-upregulated modulator of apoptosis) and Bax (Bcl-2-associated X protein) act as autophagy stimulators. It has been discovered that the protein PUMA, which is only found in the mitochondria, induces mitochondrial autophagy. This function of PUMA is distinct from the function of autophagy induced by starvation or ER stress, which is dependent on the presence of the Bax or Bak proteins. Additionally, mitochondrial-selective autophagy can be induced in the absence of PUMA activation in the presence of only Bax (Yee et al., 2009). These pro-apoptotic genes are likely to induce apoptosis and autophagy in a manner that is closely related. Through its direct physical interaction with the BCL-xL receptor, the p53-regulated tumor suppressor protein p14ARF (alternate reading frame protein product of the CDKN2A locus) appears to be able to induce autophagy in human cancer cells (Pimkina et al., 2009; Balaburski et al., 2010). It has been recently confirmed that p14ARF's tumor suppressive properties are achieved through autophagy activation (Verma et al., 2021). Additionally, the same report resolved previous discrepancies between two p14ARF mRNA isoforms and demonstrated that

autophagy can only be induced by the full-length p14ARF mRNA in the nucleus, while mitophagy is induced by smARF (selective macroautophagy of mitochondria) (Ueda et al., 2008; Budina-Kolomets et al., 2013). Studies have found that p53-mediated autophagy begins with DAPK-1 stimulation, with increased gene expression as a secondary response (Zalckvar et al., 2009b). In order to carry out autophagy, DAPK-1 uses two different routes. In the one instance, Beclin-1 phosphorylation inhibits the BCL-2/BCL-xL-mediated degradation of Beclin-1, while in the other, LC3-interacting MAP1B inhibition keeps autophagy from proceeding (Harrison et al., 2008; Zalckvar et al., 2009a).

It has been reported that in $p53^{-/-}$ cells, only the cytoplasmic p53 can inhibit autophagy through suppressing AMPK and inducing mTOR, resulting in the hyperphosphorylation of AMPK, TSC2, and acetyl CoA carboxylase (ACC) and hypophosphorylation of mTOR substrate, p70S6K (Tasdemir et al., 2008a). Autophagy in HCT116$p53^{-/-}$colon carcinoma cells is reduced when they are re-transfected with the p53 wild-type allele. Furthermore, when transfected into $p53^{-/-}$ cells, p53 mutants that preferentially localize to the cytoplasm are found to effectively repress autophagy (Morselli et al., 2008). According to all of these observations, it is evident that p53 in the cytoplasm inhibits autophagy. It has been previously shown that TIGAR (TP53-induced glycolysis and apoptosis regulator) has a molecular link to p53's anti-autophagic function (Bensaad et al., 2006). Under stressful conditions, inhibition of autophagy by TIGAR, which is a direct target gene of the tumor suppressor gene p53, has been shown to be associated with downregulation of glycolysis and suppression of ROS formation (Bensaad et al., 2009). When TIGAR's function is impaired, ROS levels increase, triggering autophagy induction. Nevertheless, it is unlikely to have an effect on the mTOR pathway (Tang et al., 2021a). It is most likely to have a non-mTOR-mediated metabolic pathway as TIGAR does not appear to have a significant impact on mTOR signaling. The interaction of p53 in embryonic carcinoma cells with Beclin-1 leads to the ubiquitination and degradation of the p53, which thus suppresses autophagy (Tripathi et al., 2014). By inhibiting cytoplasmic p53, this effect can be reversed, and autophagy can be induced more effectively.

Cancer cells acquire unique metabolic characteristics to ensure their survival and proliferation (DeBerardinis, 2008). Recent studies have been shown that p53 regulates metabolic traits of cells in addition to its role as a tumor suppressor protein (Wen and Wang, 2021), but the exact mechanism by which p53 regulates metabolism is still not completely understood. As a compensatory response to protect cells against stress, increased signaling triggered by p53 leads to activation of the PtdIns3K-Akt-MAPK-Ras signaling pathway (Corcoran et al., 2006). It was suggested by Gottlieb and Vousden that p53 might be able to counteract the Warburg effect, which is characterized by an abnormally high rate of glycolysis under aerobic conditions and is seen in many cancers (Gottlieb and Vousden, 2010). Recent studies have concluded that p53-regulated metabolism and autophagy are linked which is a primary strategy for cancer treatment to manipulate autophagy regulated by the p53 gene (Shim et al., 2021). A study conducted by Buzzai *et al.* examined the effect of the anti-diabetic drug metformin on tumor growth in the presence of metformin in the colon cancer cell lines HCT116 $p53^{+/+}$ and HCT116$p53^{-/-}$, which were isogenic colon cancer cell lines. Autophagy was discovered to be activated in the presence of metformin in HCT116 $p53^{+/+}$ cells but not in HCT116 $p53^{-/-}$ cells in the presence of metformin, which contributed to the continued survival of the cells both *in vitro* and *in vivo* (Buzzai et al., 2007; Sui et al., 2011).

THERAPEUTIC ASPECT OF P53 PATHWAY MODULATION OF AUTOPHAGY IN CANCER

Recently, numerous p53-targeting treatment strategies have been established which includings dendritic cell-derived vaccines, adenoviral p53 vectors, p53-degrading E3 ubiquitin ligase inhibitors of Mdm2, and small-molecules to reinstate DNA binding activity. For example, a small molecule multi kinase inhibitor, sunitinib, has been permitted to treat metastatic renal cell carcinoma which degrade autophagic induction of wild type p53 proteins in a multiple cancer cell lines (Luo et al., 2018). Additionally, several synthetic and naturally occurring molecules have been targeted to regulate p53-mediated autophagy regulation in cancer. There are several newly discovered drugs and phytochemicals used as MDM2 inhibitors that have shown potential p53-mediated cancer preventive activities *in vitro* and *in vivo*. This section will focus on their efficacy and mechanisms of action.

Synthetic Drug Targeting p53-Mediated Autophagy Modulation in Cancer

Several synthetic chemicals have been used to modulate p53-mediated regulation of autophagy signaling in cancer treatment (**Table 1**, **Figure 4**). Synthetic cannabinoids was used to induce mitochondrial-mediated apoptotic and autophagy pathways in human LN18, T98G, and U251MG glioblastoma cells deficient in TP53 or PTEN tumor suppressors (Ellert-Miklaszewska et al., 2021). Gefitinib has been found to improve disease outcomes in non-small cell lung cancer (NSCLC) patients via activation of autophagy, apoptosis, senescence, and cell cycle arrest through augmenting the expression of LC3B-II, cleaved caspase-3, p21, and p53 (Zhu et al., 2015). BH3 mimetic, ABT-737, induced autophagy related protein LC-III and decreased P53 in HCT116 colon carcinoma cell lines (Tasdemir et al., 2008b). In HepG2 liver cancer cell, ABT-737 increased p62, Beclin-1, and p53 (Du et al., 2013). COX-2 inhibitor celecoxib-induced DNA damage, activated p53-dependent G-1 cell cycle arrest and regulated p53-dependent autophagy induction in human glioblastoma cells (Kang et al., 2009). Tamoxifen, a first line adjuvant endocrine therapy, was increased peptidylarginine deiminase 2 (PAD2), nuclear p53, cell cycle arrest, and apoptosis via downregulating Akt/mTOR expression in tamoxifen-resistant MCF-7 (MCF7/TamR) cells (Li et al., 2019). In contrast, Hsp90 inhibitor SNX-2112 enhanced cellular apoptosis via ROS-mediated autophagy pathway in human cervical cancer cells (Hu et al., 2019).

TABLE 1 | Several therapeutic drugs targeting p53-mediated autophagy regulation in cancer therapy.

Serial	Drugs	Model/Cancer type	Mechanism of p53 modulation	Autophagic condition	References
1	Synthetic cannabinoids	Human LN18, T98G, and U251MG glioblastoma cells	Mudulation of mutant p53	Inducetion of autophagy	Ellert-Miklaszewska et al. (2021)
2	Gefitinib	Non-small cell lung cancer (NSCLC)	Increases p53 expression	Autophagy activation	Zhu et al. (2015)
3	ABT737	HCT116 colon carcinoma cell	p53 induction	Autophagy activation	Tasdemir et al. (2008b)
4	ABT737	HepG2 liver cancer cell	Activation of p53	Autophagy induction	Du et al. (2013)
5	Celecoxib	Human glioblastoma cells U87MG and LN229 cells	p53 modulation	Induction of autophagy	Kang et al. (2009)
6	Tamoxifen	MCF-7 (MCF7/TamR) cells	Activate nuclear p53	Induction of autophagy	Li et al. (2019)
7	SNX-2112	Cervical cancer cells (HeLa cells)	p53 induction	Activates autophagy	Hu et al. (2019)
8	Temozolomide	Glioblastoma U87 cells	Modulate p53	Induction of autophagy	Lee et al. (2015)
9	Sodium selenite	Leukemia NB4 cells	Wild type p53 Modulation	Induction of autophagy	Shi et al. (2020a)
10	Metformin	HCT116 p53+/+ and p53−/− Colon cancer cell	Mudulation of p53-deficient tumor cell	Activatation of autophagy	Buzzai et al. (2007)
11	Verteporfin	Human osteosarcoma cells- HOS	p53 ubiquitinated proteins modulation	Autophagy inhibition	Saini et al. (2021)
12	Doxorubicin	Human HCC cells (HepG2, Hep3B) SNU387, and SNU449	Modulation of p53 de-ubiquitination	Autophagy regulation	Chen et al. (2021)

TABLE 2 | Numerous phytochemicals used as a therapeutic target of p53-mediated autophagy modulation in cancer.

Sl	Phytochemicals	Model/Cancer type	Mechanism of action	p53 Condition	Autophagic condition	References
1	Allicin	Hep G2 liver cancer	AMPK/mTOR/TSC2 activation	p53 level decreased	Autophagy induction.	Chu et al. (2012)
2	Sinensetin	Hep G2 human liver cancer	AMPK/mTOR. inhibition	p53 modulation	Increases autophagy	Kim et al. (2020)
3	Luteolin	HCT116. HT-29 colon cancer	Apoptosis activation	p53 level increased	Autophagy Induction	Yoo et al. (2021)
4	Quercetin	HepG2, Hep3B, MDA-MB-231, HCT116	Activation of apoptosis, TFEB, cathepsin B, cathepsin D, and LAMP-1	p53 level increased	Autophagy induction.	Wang et al. (2021b)
5	Resveratrol	HCC human hepatocellular carcinoma cells	PI3K/Akt and Beclin1, LC3 II, and p62 activation	p53 level increased	Autophagy induction.	Zhang et al. (2018)
6	Mimulone	Human A549, MCF-7, HCT116, U2OS cells	AMPK/mTOR activation	p53 level decreased	Induction of autophagy	An et al. (2014)
7	Diosmin	MCF-7, MDA-MB-231, SK-BR-3 cell	p21, p27, and ERK1/2 activation	p53 level increased	Autophagy induction.	Lewinska et al. (2017)
8	Honokiol	Human U87 MG glioma cells	Akt/mTOR downregulation	p53 induction	Autophagy induction	Lin et al. (2016)
9	Oridonin	HCT-15, COLO205, HCT116, RKO, SW480, and SW620	AMPK deactivated autophagy induction	p53 decresed	Induction of autophagy	Yao et al. (2017)
10	Physapubescin B	HeLa and HCT116	mTORC1 and ROS suppression	p53-dependent	Autophagy inhibition	Xu et al. (2017)
11	Sulforaphane	Malignant mesothelioma (H-28)	Akt/mTOR reduction	p53 level increased	Induction of autophagy	Lee and Lee, (2017)
12	A-24	p53 wild-type and-deficient gastric cancer cells	PI3K/Akt/mTOR pathway	p53 modulation	Autophagy induction	Xu et al. (2021)

Temozolomide has been found to induce autophagy and p53 as well as phospho-p53 levels in glioblastoma U87 cells (Lee et al., 2015). Moreover, sodium selenite induced autophagy and apoptosis in p53 wild type cells without caspase-8/apoptosis-inducing factor activation and upregulated PLSCR1 in Leukemia NB4 cells (Shi K. et al., 2020). It has been reported that anti-diabetic drug, metformin, activated autophagy via mTOR inhibition and AMPK activation in p53-deficient tumor cell growth of cancer HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cell lines (Buzzai et al., 2007). Furthermore, metformin inhibited matrix metalloproteinase-9 activation, decreased endogenous insulin resistance, suppressed HER2 (erbB-2) oncoprotein overexpression, improved cancer patient's survival in type 2 diabetes, and blocked migration as well as invasion of cancer cells (Sui et al., 2011). Recently, Saini *et al.* found that verteporfin, known as autophagy inhibitory and proteotoxic functions,

disrupts multiple steps of autophagy in addition to regulate p53 to sensitize osteosarcoma of human osteosarcoma cells- HOS (R156P mutant P53) (Saini et al., 2021). microRNA, miR-26b, improves the sensitivity of hepatocellular carcinoma to doxorubicin by USP9X-dependent degradation of p53 as well as autophagy regulation (Chen et al., 2021).

Phytochemicals/Natural Products Targeting p53-Mediated Autophagy Regulation in Cancer Therapy

Phytochemicals from edible as well as medicinal plants have shown to potent cancer chemotherapeutic and chemopreventive activities. Several phytochemicals have mediated their anticancer properties via targeting p53 (Qin et al., 2018) (**Figure 5**). Numerous phytochemicals/natural products have been used to modulate p53-mediated autophagy pathways as a therapeutic target are presented in **Table 2**. Allicin reduced cytoplasmic p53, Bcl-2, and inhibited PI3K/mTOR signaling pathway in addition to increase AMPK/TSC2 and Beclin-1 expression in Hep G2 cells (Chu et al., 2012). Sinensetin-mediated autophagy has been involved in p53-induced AMPK/mTOR signaling pathway in HepG2 Cells (Kim et al., 2020). In p53 wild, HCT116 cells, luteolin exhibited anti-cancer effects via the regulation of p53 through cell cycle arrests such as PARP/p21 and apoptosis mediated by Nova and Bax (Yoo et al., 2021). Quercetin, a flavonoid derived from fruits and vegetables, was found to induce p53-independent/mTORC1 mechanism in various cancer cells such as human hepatocellular carcinoma cells (HepG2, Hep3B, MDA-MB-231) and colorectal cancer cells (HCT116, GFP-LC3 Hela cells) (Wang Z. X. et al., 2021). It has been demonstrated that resveratrol inhibited pAkt/Akt and induced autophagy related protein Beclin-1, LC3-II and p62 in HCC cells (Zhang et al., 2018). The anticancer mechanism of mimulone has been mediated by an increase of specific markers of autophagy such as LC3-I and LC3-II along with inhibition of p53, *p*-mTOR and increase of *p*-AMPK (An et al., 2014). Diosmin, derived from citrus fruits, has been identified as a mediator of oxidative and nitrosative stress caused by DNA damage and DNA methylation lead to G2/M cell cycle arrest, elevation in p53, p21, p27 and ERK, mediated by autophagy (Lewinska et al., 2017). Honokiol, a lignan belonging to the genus *Magnolia*, induced ROS-mediated autophagic cell death via regulating the p53/PI3K/Akt/mTOR signaling pathway in human U87 MG glioma cells (Lin et al., 2016). Oridonin, a natural diterpenoid isolated from the traditional Chinese herb, activated autophagy through inhibition of glucose metabolism and AMPK inhibition in p53-mutated colorectal cancer cell (Yao et al., 2017). A steroidal compound, physapubescin B, extracted from *Physalis pubescens* L. (Solanaceae), has been described to possess anti-cancer potential through excessive ROS generation and induce p53-dependent apoptotic cell death by autophagy inhibition in cervical cancer (HeLa) and colon cancer (HCT116) cells (Xu et al., 2017). Sulforaphane (SFN), an isothiocyanate compound found in cruciferous vegetables, potentiates apoptosis and promotes autophagy in malignant mesothelioma cells via activation of p53 (Lee and Lee, 2017; Uddin MS. et al., 2020). A steroidal saponin, A-24, derived from *Allium chinense*, induced apoptosis and autophagy along with migration inhibition in p53 wild-type as well as p53-deficient gastric cancer cells via ROS accumulation in independent of p53 (Xu et al., 2021).

Perspectives and Limitations of p53-Modulated Autophagy Cancer Therapy

The role of p53 in autophagy regulation in cancer progression has established into a strongly knit, exciting, and rapidly changing disciple in biological science. However, the study of the ability of p53 to modulate autophagy in addition how this modulation of regulation of cancer metabolism raises numerous issues. The basic process of autophagy is important for normal cellular function as its dysregulation is generally encountered during human tumor development (Yan and Chen, 2021). However, p53 and autophagy comprise a two-edged sword as well as possess an important function in tumor development and progression (Thorburn, 2014; Gao et al., 2020). Depending on the cancer type and entity, p53 and autophagy molecular predisposition in relation to tumor mutations, both can either encourage or inhibit tumorigenesis (Mrakovcic and Frohlich, 2018). There is currently not much evidence of p53-mediated autophagy regulation in cancer metabolism. Recently, it has been found that p53 activates cell cycle arrest in MEFs cell, whereas it induces apoptosis in oncogene-transformed MEFs cell which indicates that p53 exerts its tumor inhibition function in a cell- and tissue-dependent manner (Kon et al., 2021). Meanwhile, autophagy activation leads to clearance of subcellular organelle, or autophagic cell atrophy, or autophagic cell death in which tumor suppression occurs upon activation of p53 in a certain type of tissue (Jin, 2005). Additionally, autophagy activation contributes to determining cell fate upon p53 activation (Chen, 2016). However, autophagy downregulation either via mutations of autophagic genes, or activation of mTOR signaling through the activation of an abnormal oncogene might change p53-mediated apoptosis or necrosis with cell cycle arrest (Denisenko et al., 2018). Forthcoming studies would be required to investigate the epigenetic and genetic modifications of autophagy pathway in cancer in the context of p53 tumor suppression. p53 network and mTOR network will not only provide a new understanding of tumorigenesis, but also provide a clue for the target of cancer chemotherapy. Accordingly, the function of normal p53 might be compromised. Furthermore, the consequences of autophagy regulation via p53 modulation for cancer prognosis are quite difficult to predict. The link between p53 and autophagy provide a novel mechanism which p53 might play an important functional role as a guardian of metabolic balance in cancer suppression. These new functional role of p53-mediated autophagy modulation would be provided an interesting potentials for the development of novel cancer therapies.

CONCLUSION

The role and impact of modulation of p53 in regulation of autophagy is complex and far from fully clarified. Emerging evidence and rapidly developed omics as well as genome editing techniques have likely been to revolutionized a new p53 roles in autophagic activities of different p53 proteins may vary along with changes in tumor microenvironment. Therefore, novel technologies may shed a new perceptions for a knowledge-based insights to recognize gaps-existing knowledge in addition to analyze scenarios which involve a reconsideration for the function of p53 modulation in autophagy signaling in cancer. Recently, autophagy has been established as a dual role in tumor suppression process likely involved in human cancer research. p53 might be an essential player in the modulation of autophagy pathway, although the exact molecular mechanisms and cellular function in cytoplasmic and nuclear p53-mediated autophagy regulation have not been well studied. However, cellular function and role of p53-mediated autophagy, as well as molecular metabolism in cancer progression, require a strongly related and rapidly altering field. The regulation of cancer metabolism by p53 target genes can diverge according to the stress signal, cell type, and other conditions. Additional, it is evidently established that p53 stabilization is a tumor-specific vulnerability, approaches to indorse the degradation of p53 through autophagy which represents an attractive anti-cancer method. Nevertheless, our augmented understanding of the function of p53 and autophagy will hopefully offer a prospective approach to cancer treatment. Therefore, this review revealed that p53 could be targeted as an important implication of cancer therapy via modulation of autophagy signaling. Hitherto the actual therapeutic use of p53-mediated autophagy induction needs detailed knowledge of how the autophagy-lysosomal pathway may affect in cancer progression.

AUTHOR CONTRIBUTIONS

Idea and conceptualization by MAR. Figures are drawing by MHR. Writing and original draft preparation by MNP, MMMR, RI, MJU, and MAH. Visualization and supervision by BK. All authors have read and agreed to the published version of the manuscripts.

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REVIEW
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It's Getting Complicated—A Fresh Look at p53-MDM2-ARF Triangle in Tumorigenesis and Cancer Therapy

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Anti-tumorigenic mechanisms mediated by the tumor suppressor p53, upon oncogenic stresses, are our bodies' greatest weapons to battle against cancer onset and development. Consequently, factors that possess significant p53-regulating activities have been subjects of serious interest from the cancer research community. Among them, MDM2 and ARF are considered the most influential p53 regulators due to their abilities to inhibit and activate p53 functions, respectively. MDM2 inhibits p53 by promoting ubiquitination and proteasome-mediated degradation of p53, while ARF activates p53 by physically interacting with MDM2 to block its access to p53. This conventional understanding of p53-MDM2-ARF functional triangle have guided the direction of p53 research, as well as the development of p53-based therapeutic strategies for the last 30 years. Our increasing knowledge of this triangle during this time, especially through identification of p53-independent functions of MDM2 and ARF, have uncovered many under-appreciated molecular mechanisms connecting these three proteins. Through recognizing both antagonizing and synergizing relationships among them, our consideration for harnessing these relationships to develop effective cancer therapies needs an update accordingly. In this review, we will re-visit the conventional wisdom regarding p53-MDM2-ARF tumor-regulating mechanisms, highlight impactful studies contributing to the modern look of their relationships, and summarize ongoing efforts to target this pathway for effective cancer treatments. A refreshed appreciation of p53-MDM2-ARF network can bring innovative approaches to develop new generations of genetically-informed and clinically-effective cancer therapies.

Keywords: p53, MDM2, p14ARF, ARF, *CDKN2A*, tumor suppressor, oncogene, cancer therapy

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INTRODUCTION

Discovered more than 40 years ago, tumor-suppressor p53 (encoded by *TP53* in human and *Trp53* in mouse) has become the most popular gene due to the fact that it is the most frequently altered gene in cancers (Vogelstein et al., 2010; Dolgin, 2017). Functioning as guardian of the genome, p53 responds to oncogenic stresses by inducing mechanisms like cell cycle arrest, senescence and programmed cell death (apoptosis) to allow damaged cells to either undergo necessary repairs or be eradicated from the environment before permanent transformation leading to malignant cancer progression (Kastenhuber and Lowe, 2017).

Decades of extensive studies have revealed tremendous complexity of the p53 universe. A master regulator of systemic homeostasis, p53 regulates pathogenesis of many diseases other than cancer, including neurodegeneration, cardiovascular diseases, metabolic disorders, autoimmune and infectious diseases (Takatori et al., 2014; Siegl and Rudel, 2015; Kung and Murphy, 2016; Szybinska and Lesniak, 2017; Aloni-Grinstein et al., 2018; Maor-Nof et al., 2021; Men et al., 2021). As if we need a further reminder about p53's significance in human health, the culprit of the global COVID-19 pandemic, SARS-CoV-2, also targets p53 for its full pathogenic effects (Cardozo and Hainaut, 2021). Connections between p53 and these diverse physiological conditions led to expanded knowledge of many biological processes downstream of p53, such as metabolism, autophagy, translational control and epigenetic regulation, among others (Levine, 2019; Boutelle and Attardi, 2021).

Equally complicated is the network of mechanisms regulating p53 functions. Regulation of p53 is dictated by many factors, including mutation status and post-translational modification of p53, composition of response elements (REs) of p53 target genes, interaction between p53 and cofactors, and the heterogeneity in spatial and temporal dynamics of p53 activity (Hafner et al., 2019; Farkas et al., 2021). It is a highly choreographed process to control cell fate through the huge number (>3,500 by estimation) of p53 target genes and other p53-controlled mechanisms (Fischer, 2017; Sammons et al., 2020).

Amidst the tremendous complexity surrounding p53, one constant is the central hub formed by p53 and its key regulator, mouse double minute 2 (MDM2). The relationship between p53 and MDM2 is considered the final gatekeeper for majority of stress-induced signaling pathways whose main objective is to unlock the power of p53-mediated activities (Levine, 2020). The importance of p53-MDM2 hub also signifies the critical roles of direct MDM2 regulators, chief among them alternate open reading frame (ARF), in controlling p53 functions. We will herein summarize the conventional understanding of p53-MDM2-ARF relationships, unconventional and unique perspectives provided by recent studies, and implications for cancer therapeutics as our knowledge of this powerful triangle continues to evolve.

THE SIMPLE TRIANGLE CONNECTING P53, MDM2 AND ARF

Conventional Wisdom for p53-MDM2-ARF Relationship

To deploy anti-tumorigenic functions, wild type (WT) p53 stands ready to be activated in short orders, while maintaining in the background of cellular machineries to prevent unnecessary damages. This fast-deployment system requires a simple mechanism for on and off switches, controlled mainly by a single protein, MDM2. Initially recognized as an oncogene overexpressed in transformed mouse cells, MDM2 was quickly found to promote tumorigenesis by inhibiting p53's transcriptional activity (Fakharzadeh et al., 1991; Cahilly-Snyder et al., 1987; Oliner et al., 1993). The structure of MDM2 contains a main N-terminal p53-binding domain, a C-terminal RING domain and sequence motifs facilitating its localizations in (NLS: nuclear localization signal; NoLS: nucleolar localization signal) and out (NES: nuclear export signal) of nucleus (**Figures 1A,B**). The interaction between MDM2 and p53 is made particularly strong by p53's ability to bind MDM2 through multiple interfaces (Chi et al., 2005; Yu et al., 2006; Poyurovsky et al., 2010). Functioning as a E3 ubiquitin ligase, MDM2 serves as a constant quencher of p53 activity by mediating ubiquitination of p53 on its C-terminus to promote proteasome-mediated degradation (Haupt et al., 1997; Midgley and Lane, 1997). To release the strong clamp of MDM2, stress-induced signaling pathways use a variety of mechanisms to probe and prod between p53 and MDM2 to free p53. These mechanisms mainly lead to post-translational modifications (PTM) of p53, such as phosphorylation at serine 15/20/37/106 and threonine 18 to weaken p53-MDM2 interaction, and acetylation at C-terminal domain (CTD) lysine residues to prevent MDM2-mediated ubiquitination (Shieh et al., 1997; Unger et al., 1999; Nakamura et al., 2000; Rodriguez et al., 2000; Sakaguchi et al., 2000; Li et al., 2002; Hsueh et al., 2013). The relative significance of these PTM events has been a subject of debates. For example, lysine-to-arginine (KR) substitutions at multiple CTD acetylation sites significantly altered expression of p53 target genes but resulted in few abnormal phenotypes in mouse models (Krummel et al., 2005; Tang et al., 2008). A nonsense mutation at serine 15 was found to reduce p53-mediated transactivation but have little effect on p53's interaction with MDM2 and its stability (Dumaz and Meek, 1999). These discrepancies can be attributed to functional regulation of individual PTM sites, crosstalk between PTM sites, other MDM2-mediated p53 PTM such as neddylation, and complexity surrounding p53-MDM2 hub to calibrate p53 activities (Lambert et al., 1998; Xirodimas et al., 2004; Laptenko et al., 2015). To ensure that p53 functions are only activated in a transient manner, MDM2 is transcriptionally induced by WT p53 to form a regulatory feedback loop (Barak et al., 1993). p53-mediated regulation of MDM2 likely contributes to a system capable of fine-tuning p53 functions.

Another way to activate p53 functions is through direct inhibition of MDM2. Among pathways reported to date, ARF-mediated MDM2 inhibition is the most well studied mechanism. ARF (or p14 in human and p19 in mouse) is encoded by the *CDKN2A* locus, which also encodes another tumor suppressor, p16INK4A (**Figure 1C**). ARF and p16INK4A are transcribed from two partially overlapped open reading frames and translated to two unrelated proteins. ARF activates p53 by directly interacting with MDM2 to inhibit its functions (Kamijo et al., 1998; Pomerantz et al., 1998). Mechanistically, two arginine rich domains (amino acids, or aa 1–14 and 82–101) of ARF predispose its localization to the nucleolus (Zhang and Xiong, 1999; Rizos et al., 2000). The N-terminal 1–14 motif interacts with the central region of MDM2, exposing its NoLS motif to sequester ARF-MDM2 complex in the nucleolus (Weber et al., 1999; Weber et al., 2000a; Lohrum et al., 2000). This phenomenon prevents MDM2 from exporting p53 into the cytoplasm for degradation, thus preserving p53 functions

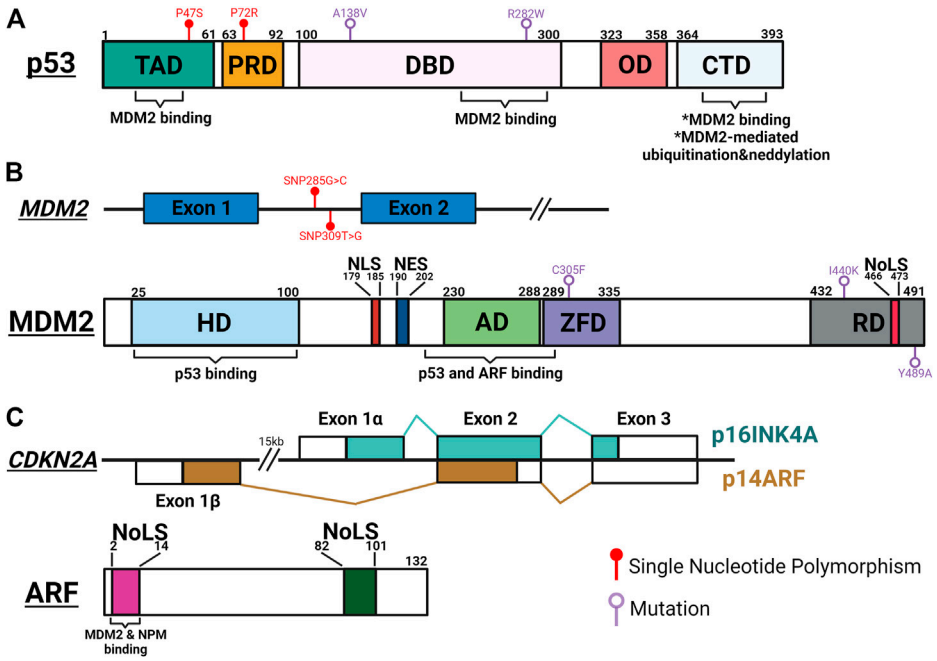

FIGURE 1 | Schematic summary of important genetic and protein features of p53, MDM2 and ARF. **(A)** p53 interacts with MDM2 through its transactivation domain (TAD), DNA binding domain (DBD) and carboxy-terminal domain (CTD). MDM2-mediated post-translational modifications occur at the CTD, leading to inactivation and degradation of p53. Single nucleotide polymorphisms (SNP) in TAD (P47S; rs1800371) and proline-rich domain (PRD) (P72R; rs1042522) modulate p53's ability to suppress tumorigenesis and regulate metabolic fitness. Temperature sensitive mutations of p53 in DBD (A138V and R282W) result in resistance to MDM2-mediated degradation. OD, oligomerization domain. **(B)** Upper panel: SNPs of *MDM2* regulate the functional oscillation between p53 and MDM2. SNP309T>G (rs2279744) results in higher MDM2 expression and inhibits p53-MDM2 oscillation. SNP285G>C (rs117039649) contributes to lower MDM2 expression and is associated with reduced risks for female reproductive cancers. Lower panel: MDM2 interacts with p53 through its N-terminal hydrophobic domain (HD) and acid domain (AD), and with ARF through AD. Interaction with ARF exposes the NoLS motif in the RING domain (RD) to sequester the ARF-MDM2 complex in the nucleolus. A cancer-associated single mutation, C305F, in the zinc finger domain (ZFD) mediates interaction between MDM2 and ribosomal proteins (RP) to regulate p53 function in response to metabolic stress. Two mutations in RD (I440K and Y489A) reduce MDM2-mediated p53 degradation but still limit p53 activity in response to DNA damage. **(C)** Upper panel: The *p16INK4A/p14ARF* locus. Each transcript utilizing a unique first exon, *p16INK4A* (Exon 1α) and *p14ARF* (Exon 1β) splice into common exon 2 and 3 in alternate reading frames to produce two distinctive amino acid sequences, resulting in two unrelated proteins. Lower panel: ARF interacts with MDM2 and NPM through its conserved N-terminal motif between amino acids 1 and 14. Both 1–14 and 82–101 arginine rich NoLS motifs are important for ARF's ability to translocate to the nucleolus and activate p53. The figure was created with BioRender.com and not drawn to scale.

(Tao and Levine, 1999; Weber et al., 1999). In addition to the spatial restriction, ARF also stabilizes p53 through inhibiting MDM2's ubiquitin-ligase activity (Honda and Yasuda, 1999; Midgley et al., 2000). Interestingly, several studies have demonstrated disconnections between nucleolar localization of ARF-MDM2 complex, p53 stabilization, and p53-mediated functions, implicating additional complexity surrounding this linear relationship between ARF, MDM2 and p53 (Llanos et al., 2001; Korgaonkar et al., 2002). Mirroring the feed-back mechanism between MDM2 and p53, WT p53 recruits histone deacetylases (HDAC) and polycomb group (PcG) proteins to repress ARF expression (Zeng et al., 2011).

Factors Known to Function Through p53-MDM2-ARF Triangle

Mechanisms regulating the expression and function of ARF, MDM2 and p53 have been extensively studied (See reviews by Maggi et al., 2014; Hafner et al., 2019; Klein et al., 2021). In the vast pool of regulators, a few unique players function through all three to control cancer development, such as cell proliferation factor mammalian target of rapamycin (mTOR) and oncogene c-Myc (**Figure 2**). In response to cellular stresses, p53 inhibits mTOR activity either by activating mTOR inhibitor the tuberous sclerosis (TSC)1/TSC2 complex through AMP-activated kinase (AMPK) and sestrin-1/2, or inducing transcription of phosphatase and tensin homolog (PTEN) to inhibit mTOR activator AKT (Stambolic et al., 2001; Feng et al., 2005; Budanov and Karin, 2008). A recent study demonstrated, using an acetylation-defective *p53-4KR* mouse model, that p53's ability to suppress mTOR function is linked to distinctive tumor-suppressive activities independent of cell cycle arrest, senescence, and apoptosis (Kon et al., 2021). The ability of p53 to fine-tune mTOR activity has implications beyond tumor suppression. Recent studies showed that p53-regulated mTOR functions affect cells' metabolic fitness during early development and dictate evolutionary advantages/disadvantages in our ancestors (Bowling et al., 2018; Gnanapradeepan et al., 2020).

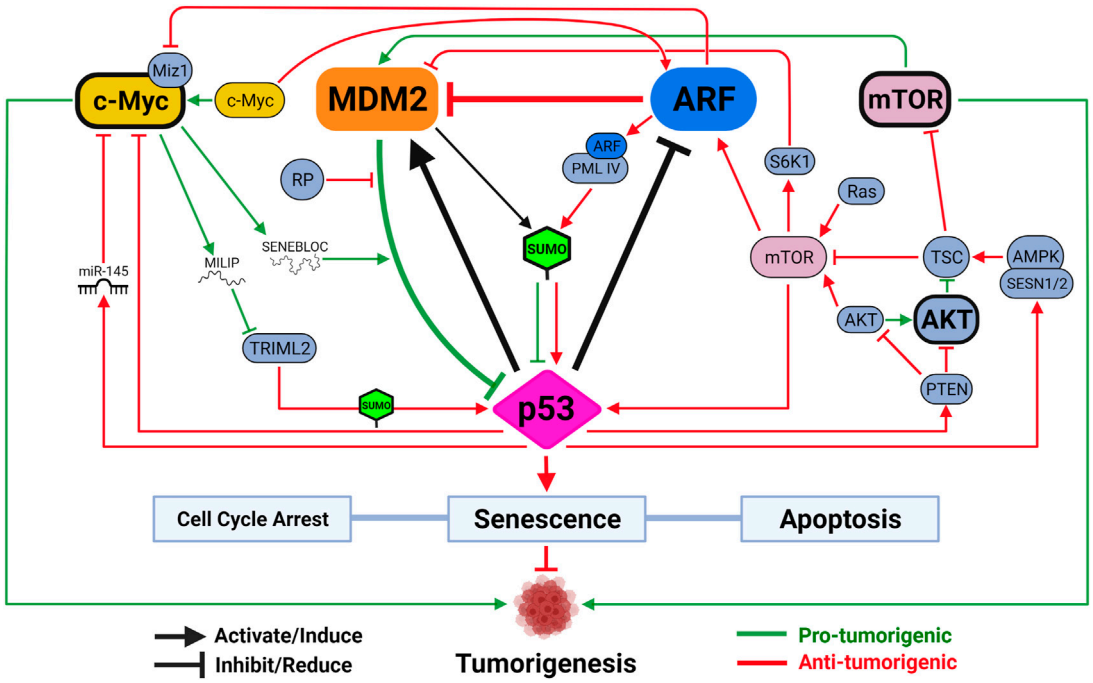

FIGURE 2 | The functional triangle between p53, MDM2 and ARF. The MDM2-p53 duplex is considered the central hub controlling p53-mediated tumor-suppressive activities. Mechanisms disrupting MDM2-p53 interaction lead to p53 activation, which induces MDM2 expression through a negative feedback loop. ARF promotes p53 activation by inhibiting MDM2. Activated p53 reduces ARF expression through HDAC and PcG transcriptional repressors, and loss of p53 often leads to ARF induction. mTOR and c-Myc are two versatile signaling factors that mediate tumor-regulating mechanisms by engaging with all three members of the triangle. Both mTOR and c-Myc (unbolded) induce p53 activation when initially encountering DNA damage stress but are both capable of, upon overexpression or sustained activation (bolded), promoting tumorigenesis by inhibiting p53 activity. Created with BioRender.com.

As a negative feedback mechanism to integrate DNA damage response into cellular metabolism, mTOR activation increases p53 activity. In the event of PTEN loss, mTOR directly binds and phosphorylates p53 to promote senescence, a phenomenon previously known to be regulated by mTOR to counter DNA damage (Korotchkina et al., 2010; Jung et al., 2019). Miceli et al. (2012) showed that, in response to oncogenic Ras signaling or loss of TSC function, activated mTOR enhances translation of existing *ARF* mRNA to promote p53 activity and tumor suppression. In cases with loss of TSC function, mTOR also induces p53 activity by activating S6 kinase 1 (S6K1) to phosphorylate MDM2 and compromise its ability to move to the nucleolus (Lee et al., 2007; Lai et al., 2010). It is worth noting, however, that excessive activation of AKT/mTOR signaling results in p53 inhibition to promote tumorigenesis in some cancers due to AKT-mediated stimulation of MDM2 (Mayo and Donner, 2001). Combined inhibition of AKT/mTOR and MDM2 in these cancers, therefore, showed some promise as a therapeutic strategy (Kojima et al., 2008; Daniele et al., 2015). A novel pro-tumorigenic activity induced by mTOR-MDM2 pathway was recently described in tumor microenvironment (TME). Kamer et al. (2020) showed that lung cancer cells induce mTOR-dependent MDM2 translation in stromal cells, establishing a positive feedback loop to promote neighboring cancer cells' metastatic potential. This mechanism was shown to be independent of stromal-p53, representing another dimension of mTOR's tumor-promoting activity.

Endogenous c-Myc induces ARF expression and p53-dependent apoptotic programs upon initial response to DNA damage, but ultimately selects for spontaneous inactivation of ARF-MDM2-p53 pathway leading to tumorigenesis (Zindy et al., 1998; Eischen et al., 1999; Nieminen et al., 2013; Phesse et al., 2014). To suppress c-Myc-induced tumorigenesis, p53 can transcriptionally repress c-Myc directly through promoting histone deacetylation or indirectly through induction of microRNA (miR)-145 (Ho et al., 2005; Sachdeva et al., 2009). ARF directly interacts with c-Myc or its transcriptional cofactor Miz1 to inactivate pro-tumorigenic transcriptional programs and induce growth arrest and cell death even in the absence of p53 (Datta et al., 2004; Qi et al., 2004; Herkert et al., 2010). Two parallel pathways through MDM2 have also been described to sustain p53 activity to counter c-Myc's pro-tumorigenic functions. In addition to ARF-MDM2 interaction, ribosomal protein (RP)-MDM2 interaction is also required to maximize p53 activity to inhibit c-Myc-induced tumorigenesis (Macias et al., 2010; Meng et al., 2015). Two recent studies demonstrated how c-Myc targets p53-MDM2-ARF tumor-suppressive axis by regulating two separate long noncoding RNAs (lncRNAs). Xu et al. (2020) identified SENEBLOC, a c-Myc-induced lncRNA involved in evasion of senescence by acting as a scaffold to increase association between p53 and MDM2, thus promoting p53 degradation. Another c-Myc

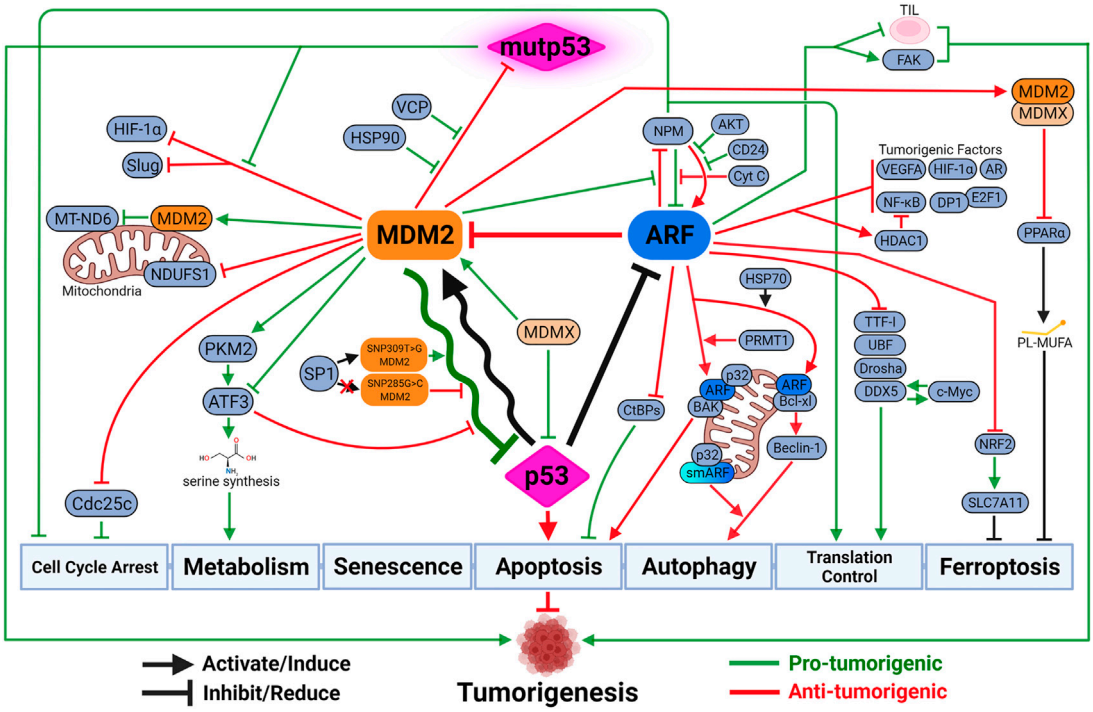

FIGURE 3 | The updated look at the p53-MDM2-ARF functional complex. Both MDM2 and ARF mediate a variety of p53-independent functions to regulate tumorigenesis. These functions can be either synergistic with or antagonizing against p53-medaited tumor-suppressive activities. Due to the negative feedback loop, the oscillatory relationship between MDM2 and p53 dictates the intensity and duration of p53-mediated tumor-suppressive activities. The outcome of the oscillation depends on their respective expression and activity levels, which are influenced by genetic alterations such as SNP and mutations. Paradoxically, MDM2 sometimes exhibits anti-tumorigenic activity by inhibiting mutant p53 (mutp53) functions, inhibiting pro-tumorigenic factors upon p53 activation, or inducing other tumor-suppressive mechanisms like ferroptosis. ARF also possesses tumor-promoting capabilities when protecting cancer cells against specific types of cell death like anoikis, or exhausting functions of tumor-infiltrating lymphocytes (TIL) to promote cancer progression. Created with BioRender.com.

responsive lncRNA, c-Myc-Inducible Long noncoding RNA Inactivating p53 (MILIP), was found to promote p53 turnover by reducing p53 SUMOylation through inhibiting tripartite-motif family-like 2 (TRIML2) (Feng et al., 2020). As TRIML2 has been found to influence cell fate decisions based on duration of p53-mediated response, the exact dynamic between c-Myc and p53 could dictate outcomes of c-Myc-induced tumorigenesis, including response to different therapies (Kung et al., 2015).

Interestingly, SUMOylation of p53 has been shown as a significant PTM mechanism through which MDM2 and ARF regulate p53 functions. Both MDM2 and ARF can mediate small ubiquitin-like modifier (SUMO)-1-mediated SUMOylation of p53 through their ability to target p53 to the nucleolus (Chen and Chen, 2003). Mechanistically, ARF interacts with a specific spliced variant of promyelocytic leukemia protein (PML), PML IV, to stabilize SUMO1-conjugating enzyme UBC9 in nuclear bodies (NB) to promote p53 SUMOylation and activation (Ivanschitz et al., 2015). ARF also mediates p53-independent functions by inducing SUMOylations of other targets, including NPM and MDM2 (Xirodimas et al., 2002; Tago et al., 2005). In human cells, MDM2-ARF complex, independent of their ability to relocate to nucleolus, also promotes SUMO-2/3-mediated SUMOylation of p53 to modulate its transcriptional activity (Stindt et al., 2011). As SUMOylation is emerging as a promising therapeutic target in cancer, its interaction with p53-MDM2-ARF pathway will be under increasing scrutiny (Kroonen and Vertegaal, 2021).

THE CURIOUS CASE BETWEEN MDM2 AND P53

Evolutionarily, structural and functional features between MDM2 and p53 are highly conserved from multi-cellular eukaryotic organisms to mammals like mouse and human (Lane et al., 2010). It suggests a critical role of p53-MDM2 hub in consolidating diverse stress signaling pathways to determine cell fates. It is posited that one of the advantages of a biological central-hub like p53-MDM2 is ability to build functional complexity, including redundant, compensatory and feedback pathways, around it as needed (Levine, 2020). For example, DNA damage sensor activating transcription factor 3 (ATF3) activates p53 by preventing its degradation by MDM2, which in turn mediates ubiquitination and

degradation of ATF3 to inactivate p53 (Yan et al., 2005; Mo et al., 2010) (**Figure 3**).

Tug-of-War Oscillatory Relationship Between p53 and MDM2

Part of this complexity can be attributed to oscillatory relationship between MDM2 and p53. The regulatory feedback loop, which allows p53 to upregulate its own inhibitor MDM2, is meant to suppress lethal p53 activity in normal cells and during development (de Oca Luna et al., 1995; Jones et al., 1995; Ringshausen et al., 2006). As the result, the mutual relationship between p53 and MDM2 can dictate physiological homeostasis outside the context of cancer development. For example, normal aging process relies on a balanced p53-MDM2 signaling network, of which dysregulations lead to premature aging or pathological conditions (Wu and Prives, 2018). Interestingly, it has been shown that p53 oscillates faster in mouse and rat cells than in cells from human, monkey, or dog. It is suggested that faster p53 oscillations in mouse might be due to subtle changes in p53 RE of mouse MDM2, leading to altered expression of MDM2 and a stronger feedback loop signal (Stewart-Ornstein et al., 2017). These variations could have significant consequences, due to the connected nature between p53-MDM2 oscillations and transcriptional regulations of p53 target genes (Hafner et al., 2017). In cancer cells, it is suggested that oscillatory p53-MDM2 activity is dictated by the intensity of stress signals, as well as expression level of p53 and MDM2 upon encountering stresses (Lev Bar-Or et al., 2000; Lahav et al., 2004; Ma et al., 2005). This hypothesis was demonstrated in cells with a single nucleotide polymorphism (SNP) of *MDM2* (SNP309T>G; rs2279744) that results in higher level of MDM2 and inhibition of coordinated p53-MDM2 oscillation (Hu et al., 2007). Higher expression of SNP309T>G *MDM2* is due to increased affinity of its transcriptional activator Sp1, which preferentially responds to estrogen signaling (Phelps et al., 2003; Bond et al., 2004). As the result, SNP309T>G *MDM2* is found to associate with accelerated tumor formation in a gender-specific and hormone-dependent manner (Bond et al., 2006; Post et al., 2010). In contrast, another *MDM2* SNP that is only 24 base pairs upstream of SNP309, SNP285G>C (rs117039649), disrupts an Sp1-binding site to decrease MDM2 expression. SNP285G>C is exclusively found in Caucasians and, when coexisting with SNP309T>G, associates with reduced risks for female reproductive cancers (Knappskog et al., 2011). Interestingly, increased longevity was observed in females with SNP309T>G *MDM2* if they didn't suffer from cancer diagnoses (Gross et al., 2014). This phenomenon could be attributed to higher MDM2 expression leading to suppressed p53 stress response in stem cell populations. Similar paradoxical regulations between cancer susceptibility and metabolic fitness have been linked to other SNPs in p53-MDM2 pathway, including Proline72Arginine (P72R; rs1042522) and Proline47Serine (P47S; rs1800371) of p53 (Kung et al., 2015; Jennis et al., 2016; Kung et al., 2016; Kung et al., 2017; Gnanapradeepan et al., 2020). It remains to be seen if these SNPs also impact the fine balance separating tumorigenesis and homeostasis through regulating oscillatory activity between p53 and MDM2.

Tumor-Suppressive Functions of MDM2

Negative feedback activity is not the only outcome for p53-mediated MDM2 induction. For example, in non-small-cell lung cancer (NSCLC), WT p53 suppresses cancer metastasis by facilitating MDM2-mediated degradation of metastatic promoter Slug (Wang et al., 2009). More recently, p53-induced MDM2 was found to slow down cell cycle progression by promoting degradation of mitosis-promoting factor Cdc25C, which is also a transcriptionally-repressed target of p53 (Clair et al., 2004; Giono et al., 2017). Considering that MDM2 can potentially reach many targets through its E3 ligase activity, the consequence of p53-induced MDM2 expression could be tumor-promoting or tumor-suppressing depending on the cell type and surrounding factors. Hypoxia-inducible factor 1-alpha (HIF-1α) is another pro-tumorigenic factor that can be degraded by MDM2 in a p53-dependent manner (Ravi et al., 2000). Interestingly, mutant p53 (mutp53) was found to exert its tumor-promoting activity by dissociating HIF-1α from MDM2, leading to HIF-1α upregulation (Kamat et al., 2007). The aforementioned metastatic promoter Slug can also be stabilized in the presence of mutp53, which represses MDM2 through inhibiting p73-mediated MDM2 transactivation (Wang et al., 2009). MDM2 can also mediate the degradation of mutp53 and keep it at basal levels in cancer cells (Haupt et al., 1997; Terzian et al., 2008). Since mutp53 is incapable of inducing MDM2 expression to complete the feedback loop, it can be stabilized through interacting with factors disrupting mutp53-MDM2 complex, such as heat shock protein (HSP) chaperones HSP90 and valosin-containing protein (VCP), to execute pro-tumorigenic activities (Midgley and Lane, 1997; Peng et al., 2001; Li et al., 2011a; Wang et al., 2021). The recent finding demonstrating functional plasticity of mutp53 between tumor-suppressor and tumor-promoter revealed new dimension in p53 biology, including MDM2's potential role in regulating mutp53 activities (Kadosh et al., 2020).

MDM2 Functions Antagonizing Against or Synergizing With p53 Activity

Not surprisingly, functional complexity evolving around MDM2 has drawn increasing attention in recent years (Klein et al., 2021). Many MDM2-mediated functions have been shown to operate independent of p53 but demonstrate capacities to synergize or antagonize p53-mediated pathways. It is well established that mitochondria p53 confers important biological functions, both in mediating mitochondria-based apoptosis and regulating mitochondrial respiration to control cancer development (Leu et al., 2004; Murphy et al., 2004; Matoba et al., 2006). It has been shown that upon oxygen deprivation, a fraction of MDM2 localizes to the mitochondria in p53-independent manner, inhibits mitochondrial respiration by reducing complex I subunit NADH-dehydrogenase 6 (MT-ND6), enhances

reactive oxygen species (ROS) production, and promotes cancer cell migration and invasion (Arena et al., 2018). Interestingly, however, a more recent study showed that cytosolic MDM2, by sequestering mitochondria stabilizer NADH:ubiquinone oxidoreductase 75 kDa Fe-S protein 1 (NDUFS1), induces ROS to promote apoptosis (Elkholi et al., 2019). It remains to be seen what regulatory mechanisms differentiate MDM2's ability to promote or inhibit tumorigenesis through regulating mitochondria functions.

Under metabolic stress, p53 can support cancer cell proliferation and survival by mediating metabolic reprogramming. One such mechanism manifests in the event of serine deprivation, during which p53 activates the synthesis of serine and glutathione, preserving anti-oxidant activity to reduce oxidative stress (Maddocks et al., 2013). MDM2 is also capable of triggering serine synthesis pathway upon serine starvation, independent of p53, through PKM2 (pyruvate kinase 2)-mediated recruitment to chromatin to facilitate a ATF3/4-mediated transcriptional program (Riscal et al., 2016). It will be interesting to dissect the regulatory mechanisms distinguishing this pathway and aforementioned p53-MDM2-ATF3 feedback loop upon DNA damage. In contrast to the pro-tumorigenic functions in response to serine depletion, MDM2 and p53 can also converge on anti-tumorigenic pathways, such as an iron-dependent form of nonapoptotic cell death, ferroptosis (Stockwell et al., 2017). Jiang et al. (2015) first showed that ferroptosis is a critical mechanism for p53-mediated tumor suppression. Their argument relies on the fact that an acetylation-defective p53 mutant, p53(3KR), retains ferroptosis-inducing and tumor-suppressing capabilities despite failing to promote cell-cycle arrest, senescence and apoptosis. This is supported by the discovery that a African-centric, cancer-predisposing p53 polymorphism P47S has impaired ability to promote ferroptosis by inducing levels of antioxidants coenzyme A (CoA) and glutathione (GSH) (Jennis et al., 2016; Leu et al., 2019). Interestingly, Liu et al. (2017a) showed that mutp53 can sensitize some cancer cells to ferroptosis by inhibiting the cystine/glutamate antiporter and glutathione biosynthesis, providing another mechanistic basis for p53 reactivation therapy. A recent finding by Venkatesh and colleagues showed that MDM2, working in a complex with Murine Double Minute X (MDMX), facilitates ferroptosis through altering cellular lipid profiles and preventing anti-oxidant responses (Venkatesh et al., 2020). Interestingly, MDM2's positive regulation of ferroptosis may not be entirely p53-independent. It was shown in some cancer cells, stabilization of p53 by MDM2 inhibitor Nutlin-3 delays ferroptosis induced by cystine deprivation (Tarangelo et al., 2018). This phenomenon was found to depend on the p53 target gene p21, but the underlying mechanism is still unclear. Whether this effect is dictated by the p53-MDM2 relationship and sensitive to other forms of MDM2 regulations requires more extensive studies.

MDMX Regulates MDM2-p53 Functions

Despite the recent discovery of its role collaborating with MDM2 to promote ferroptosis, MDMX (also known as MDM4) is mostly considered a pro-tumorigenic factor like MDM2 (Ramos et al., 2001). Similar to MDM2, MDMX can directly inhibit p53 functions through binding between their N-terminal domains (Shvarts et al., 1996; Danovi et al., 2004). The functional significance of MDMX-mediated p53 inhibition was demonstrated in a transgenic mouse model where loss of *Trp53* rescues embryonic lethality caused by *Mdm4* deletion (Parant et al., 2001).

In addition to inhibiting p53 functions directly, MDMX's contribution to tumorigenesis could also be attributed to its ability to enhance MDM2 activity. Although MDMX does not possess intrinsic E3 ligase activity, early investigations showed that it can form heterodimers with MDM2 to increase MDM2 stability and promote MDM2-mediated ubiquitination and degradation of p53 (Sharp et al., 1999; Gu et al., 2002; Linares et al., 2003). Subsequent studies revealed that the C terminus of MDMX not only is required for the formation of MDM2/MDMX heterodimer, but also is able to rescue E3 ligase activity lost in MDM2 containing E3-defective C-terminal mutations (Singh et al., 2007; Uldrijan et al., 2007). Moreover, it was later shown that MDM2/MDMX heterodimer is a more efficient E3 ligase of p53 compared to MDM2 homodimer, suggesting that it could be the predominant form in cells regulating p53 functions (Kawai et al., 2007; Huang et al., 2011; Leslie et al., 2015). The functional relationship between p53, MDM2 and MDMX is evolutionarily conserved, highlighting their importance in maintaining physiological homeostasis (Momand et al., 2011; Dolezelova et al., 2012; Coffill et al., 2016). Interestingly, MDMX's E3 ligase activity was found to be retained in some invertebrates and can be restored in the human ortholog by substituting a few amino acids (Iyappan et al., 2010; Coffill et al., 2016). It suggests that functions of MDMX have evolved to adapt to increasing environmental complexities, potentially through its interactions with MDM2 and p53 (Tan et al., 2017).

THE EXPANDING UNIVERSE BETWEEN ARF AND P53

The importance of ARF in tumor suppression was readily demonstrated in mouse models. Transgenic mice homozygous for *Arf* loss ($p19Arf^{null}$) succumb to spontaneously-developed tumors of a wide spectrum, including sarcomas, lymphomas, carcinomas and nervous system cancers, within a year (Kamijo et al., 1997; Kamijo et al., 1999). Functional distinctions between Arf and p16Ink4a were also evident in mice, in which loss of both tumor suppressors results in significantly more severe phenotypes (Sharpless et al., 2004). The picture of ARF's functional significance in human cancers is murkier. Despite the loss of *CDKN2A* being the most frequent genetic event second only to p53 mutations, it is difficult to dissect the respective contributions of p14ARF and p16INK4A to tumor suppressions in human. Despite the limitations, ARF-specific alterations, both proteogenomic and epigenetic, have been found in a wide variety of human cancers including central nervous system, bladder, colon, breast, prostate, ovarian, liver,

gastric, lung, head and neck, as well as hematologic cancers. It unequivocally suggests that ARF plays a critical role in tumor suppression (Maggi et al., 2014; Inoue and Fry, 2018).

ARF Mediates p53-independent Tumor Suppression

The first indication that ARF possesses p53-independent functions was revealed when *Arf*/*Trp53* double-knockout and *Arf*/*Trp53*/*Mdm2* triple-knockout mice developed tumors of distinctive origins compared to Arf^{null} or $Trp53^{null}$ mice (Weber et al., 2000b). A similar conclusion has been reached in human cancers through demonstrations that 1) ARF is capable of suppressing tumor progression in the absence of active p53; and 2) loss of ARF often synergizes with dysregulated p53 to promote tumorigenesis (Eymin et al., 2003; Sandoval et al., 2004; Muniz et al., 2011; Forys et al., 2014). Moreover, high prevalence of *TP53* and *CDKN2A* co-inactivation has been identified in a variety of cancers, including glioblastoma, hepatocellular carcinoma, lung cancer, pancreatic cancer, bladder cancer, and triple-negative breast cancer (TNBC) among others (Cerami et al., 2012; Gao et al., 2013). It further implicates ARF's p53-independent tumor-suppressive functions, although more studies are needed to define ARF's roles apart from those of p16INK4A in each cancer type.

ARF and NPM

Several p53-independent mechanisms have been associated with ARF-mediated tumor suppression. In response to hyperproliferative signals, ARF sequesters pro-tumorigenic nucleophosmin (NPM) in nucleolus to promote cell cycle arrest (Brady et al., 2004). The relationship between ARF and NPM appears to be mutual, but the impact of NPM on ARF function is context dependent. The interaction between ARF and NPM can preserve ARF function by preventing its degradation, while overexpressed NPM or cancer-associated NPM mutants have been shown to inhibit ARF functions by restricting its ability to translocate between nucleolus and cytoplasm (Bertwistle et al., 2004; Kuo et al., 2004; Korgaonkar et al., 2005; Colombo et al., 2006; Moulin et al., 2008). This unique relationship between ARF and NPM not only can be disrupted by MDM2, but is also sensitive to other factors, including AKT, cytochrome c, and CD24 to regulate p53-dependent and -independent functions of ARF (Brady et al., 2004; Hamilton et al., 2014; Wang et al., 2015; González-Arzola et al., 2020). ARF-NPM interaction also regulates ARF's ability to promote apoptosis independent of p53 (Hemmati et al., 2002; Eymin et al., 2003).

ARF Functions at the Mitochondria

ARF-mediated apoptosis relies on ARF's ability to localize to mitochondria, and is regulated by the interaction between ARF and mitochondrial protein p32 (Itahana and Zhang, 2008). A recent study elucidated the underlying mechanism by showing that, under genotoxic stresses, PRMT1 (protein arginine methyltransferase 1) methylates arginine residues within the NLS/NoLS of ARF, resulting in the release of ARF from NPM and increased interaction between ARF and p32 (Repenning et al., 2021). Mitochondria-bound ARF induces apoptosis by activating BAK instead of BAX, suggesting a tightly-regulated process controlling ARF-mediated apoptosis in the absence of p53 (Müer et al., 2012).

BAK-dependent apoptosis is not the only anti-tumorigenic mechanism that ARF induces once reaching mitochondria. With the help of heat shock protein 70 (HSP70), ARF travels to mitochondria, interacts with Bcl-xl, disrupts the Bcl-xl/Beclin-1 complex to release autophagic factor Beclin-1 to induce autophagy (Abida and Gu, 2008; Pimkina et al., 2009; Pimkina and Murphy, 2011). This ability to induce autophagic cell death from mitochondria is interestingly shared by the full-length ARF and a shorter isoform of ARF, smARF (short mitochondrial ARF) (Reef et al., 2006; Budina-Kolomets et al., 2013). The contribution of smARF to tumor suppression remains controversial due to its low abundance and unstable nature, but its physiological function has been clearly demonstrated in a mouse model where expression of smArf significantly rescued developmental defects of *Arf*-null mice (van Oosterwijk et al., 2017). Interestingly, p32 was also found to interact with and stabilize smARF, raising the question that if p32 serves as an arbitrator at mitochondria to regulate both apoptosis and autophagy triggered by ARF and smARF (Reef et al., 2007). Both mitochondrial p32 and ARF have been shown to control metabolic programming between oxidative phosphorylation and glycolysis (Fogal et al., 2010; Christensen et al., 2014; Gotoh et al., 2018; Koss et al., 2020). Since the metabolic state of mitochondria is important in regulating both cancer cell-intrinsic and -extrinsic mechanisms, ARF's influence on tumor metabolism independent of p53 warrants further investigation (Xiao et al., 2019; Yao et al., 2019).

ARF and Translational Control

By virtue of being an nucleolar protein, ARF exerts p53-independent tumor suppression through regulating ribosome biogenesis, ribosomal RNA (rRNA) processing and translation (Sugimoto et al., 2003; Cottrell et al., 2020). ARF-mediated regulation of NPM is also involved in this process, as ARF reduces function and stability of NPM required for ribosome biogenesis (Itahana et al., 2003; Apicelli et al., 2008; Maggi et al., 2008). ARF has been shown to regulate ribosomal functions and translation through many other mechanisms, such as directly interacting with rRNA promoter, blocking nucleolar import of RNA polymerase I transcription termination factor (TTF-I), inactivating rRNA transcriptional factor upstream binding factor (UBF), downregulating rRNA-processing enzyme Drosha, and limiting nucleolar localization of RNA helicase DDX5 (Ayrault et al., 2004; Lessard et al., 2010; Saporita et al., 2011; Kuchenreuther and Weber, 2014). Interestingly, ARF's ability to interact with DDX5 also prevents interaction between DDX5 and c-Myc, disrupting a oncogenic positive feedback loop that increases c-Myc-mediated transcription and cell transformation (Tago et al., 2015).

Other p53-independent Tumor-Suppressive Functions of ARF

The reach of ARF's p53-independent, tumor-suppressive functions extends to many other cancer-related pathways. To

inhibit pro-tumorigenic machineries, ARF blocks E2F1's transcriptional activity by interacting with E2F1 and E2F1 cofactor DP1; inhibits HIF-1α-mediated transcription by sequestering HIF-1α in nucleolus; attenuates NF-κB functions by recruiting transcriptional repressor histone deacetylase 1 (HDAC1) to NF-κB subunit RelA/p65; interacts with androgen receptor to repress its transactivation activity; and suppresses translation of tumor angiogenic factor vascular endothelial growth factor A (VEGFA) (Eymin et al., 2001; Fatyol and Szalay, 2001; Martelli et al., 2001; Datta et al., 2002; Rocha et al., 2003; Datta et al., 2005; Kawagishi et al., 2010; Lu et al., 2013). ARF also interacts directly with anti-apoptotic transcriptional corepressor C-terminal binding protein 1 (CtBP1) and 2 (CtBP2), leading to their degradation and p53-independent apoptosis (Paliwal et al., 2006; Kovi et al., 2010).

ARF also promotes other anti-tumorigenic mechanisms. In response to DNA damage, ARF induces both p53-dependent and -independent senescent response, the later through ATM/ATR/CHK signaling pathway (Eymin et al., 2006; Carlos et al., 2013; Monasor et al., 2013). Another binding partner of ARF is nuclear factor erythroid 2-related factor 2 (NRF2), which transcriptionally activates *SLC7A11*, a component of the cystine/glutamate antiporter complex. By importing cystine, SLC7A11 promotes biosynthesis of antioxidant glutathione (GSH), resulting in reduction of ROS and lipid peroxides (DeNicola et al., 2011; Ye et al., 2014). By interacting with NRF2, ARF inhibits SLC7A11 expression to promote lipid peroxidation and trigger ferroptosis (Chen et al., 2017). With the list of regulated cell death mechanisms ever-increasing, more p53-independent tumor-suppressive pathways induced by ARF could be discovered in the very near future (Tang et al., 2019).

Co-Inactivation of p53 and ARF

Considering the plethora of p53-independent pathways described for ARF-mediated tumor suppression, there is surprisingly few mechanistic studies conducted in cancers with co-inactivation of p53 and ARF. Forys et al. used both mouse embryonic fibroblast (MEF) and human TNBC cell lines to show that co-inactivation of p53 and ARF induces an pro-tumorigenic signaling signature that includes induction of interferon-β (IFN-β) and activation of signal transducer and activator of transcription 1 (STAT1) (Forys et al., 2014). In a Eμ-Myc-driven lymphoma mouse model recapitulating late-stage p53 inactivation, Klimovich et al. (2019) showed that loss of ARF confers resistance to p53 restoration in established lymphoma. This result suggests that co-inactivation of p53 and ARF not only exacerbates tumorigenesis, but also compromises efficacy of p53 reactivation therapy. On the other hand, evidence is emerging to link co-inactivation of p53 and ARF to novel therapeutic opportunities. Co-deletion of *TP53* and *CDKN2A* was recently linked to gastric premalignancy and cancer progression mediated by dietary carcinogens (Sethi et al., 2020). Despite being a malignancy-driving event, co-deletion of *CDKN2A* following p53 inactivation also induces replication stress and sensitizes cancer cells to DNA damage response inhibitors. Since deletion of *CDKN2A* in this study didn't distinguish between p16INK4A and ARF, the exact contribution of ARF needs to be further studied. The same caveat is applied to the same group's another study in esophageal squamous cell carcinoma, where they found that *Trp53*/*Cdkn2a* loss synergizes with transcription factor Sox2 to promote chromatin remodeling, enhance Stat3 functions, activate endogenous retroviruses, and induce double-stranded RNA expression and dependence of RNA editing enzyme ADAR1 (Wu et al., 2021). Implication of this study could inform new strategies to develop therapies against cancers that display similar characteristics (p53/ARF co-inactivation and ADAR1 dependency), such as TNBC (Forys et al., 2014; Kung et al., 2021).

Tumor-Promoting Functions of ARF

It is worth noting that ARF's p53-independent functions have been suggested to promote cancer progression under certain circumstances. Overexpression of ARF in cancer has been mostly considered a byproduct of p53 mutation due to the previously mentioned negative feedback loop, and generally correlated with better prognosis (Kamijo et al., 1998; Silva et al., 2001; Song et al., 2014). Several studies, however, have provided mechanistic insights regarding how ARF's presence might promote progression of some cancers. Humbey et al. (2008) first described, in a mouse model, that overexpression of ARF protects Eμ-Myc-driven lymphoma by inducing autophagy in response to nutrient starvation. Their data suggests that ARF, in a tumor-type specific manner, controls the switch between cyto-toxic and cyto-protective effects of autophagy in response to metabolic stress. Another cell-intrinsic pro-survival function of ARF was shown in spreading cancer cells in which ARF interacts with focal adhesion kinase (FAK) to stabilize cytoskeleton structure and protect cells from anoikis, a form of programmed cell death during cell detachment (Vivo et al., 2017). A recent study also shed some light on a cell-extrinsic mechanism through which ARF behaves as a tumor promoter. Koss et al. showed that during cancer development, tumor-induced metabolic stress suppresses function of epigenetic modifier enhancer of zeste homolog 2 (EZH2), leading to upregulation of ARF. Without affecting p53 function, ARF promotes mitochondrial dysfunction and metabolic exhaustion of tumor-infiltrating lymphocytes (TIL), resulting in cancer progression (Koss et al., 2020). More ARF-mediated pathways, p53-dependent and -independent, are expected to be identified in regulating TME functions.

THERAPEUTIC OPPORTUNITIES TO TARGET P53-MDM2-ARF TRIANGLE

Potential therapies to activate p53 command the most attention in development of drugs targeting p53-MDM2-ARF network. Direct restoration of WT p53 expression using intra-tumoral injection of p53-delivering adenovirus has been used to treat cancers in China since 2003 (Xia et al., 2020). Extreme cautiousness towards gene therapy in general and p53-targeting gene therapy in particular casts a cloud over when this treatment will become clinically available worldwide. In contrast, tremendous amount of efforts have been devoted to develop pharmacological activators of p53, including activators of

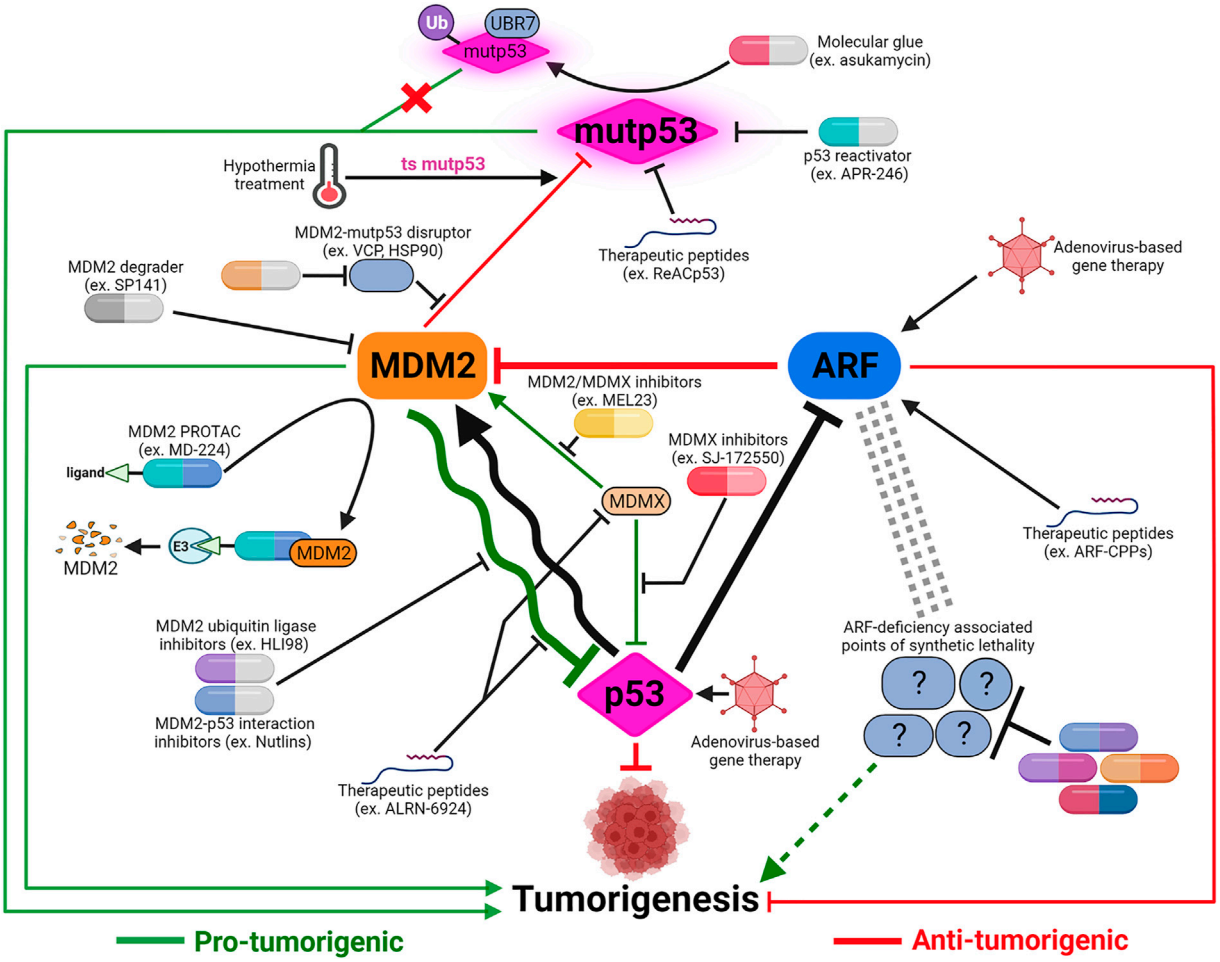

FIGURE 4 | Cancer therapeutic strategies targeting the p53-MDM2-ARF signaling axis. The majority of p53-based therapeutic strategies are designed to reactivate mutant p53 or inhibit MDM2-p53 and MDM2/MDMX-p53 interactions using small molecules. Development of alternative strategies, such as applying hypothermal therapy to induce MDM2-mediated degradation of temperature-sensitive mutp53, or utilizing pan-MDM2 inhibitors, are meant to maximize anti-tumor potency depending on the context of MDM2-p53 relationship. In addition to small molecules, other modalities including therapeutic peptides and proteolysis targeting chimera (PROTAC) are also being explored to target both MDM2's p53-dependent and -independent functions. The realization of ARF's many p53-independent functions and the functional significance of p53-ARF co-inactivation in cancers necessitates development of ARF-based therapies. Virus-mediated gene therapy and therapeutic peptides are potential ways to restore ARF functions. Identification of synthetic lethality associated with ARF deficiency can uncover novel therapeutic targets to compensate for ARF loss and potentially synergize with p53-targeting treatments. Created with BioRender.com.

WT p53 and re-activators of mutp53 to restore p53 functions (Nguyen et al., 2017). Other than PRIMA-1^{MET} (also called APR-246), a small-molecule mutp53 re-activator, most p53-targeting drugs in active clinical development/trials are small molecules that stabilize WT p53 by interrupting p53-MDM2 interaction or inhibiting MDM2's ubiquitin ligase activity (**Figure 4**).

Therapeutic Peptides for p53 Activation

Although small molecules still dominate the drug discovery landscape, other modalities have been explored as p53 activators, such as therapeutic peptides (Marqus et al., 2017). Small peptides derived from N-terminal MDM2-binding domain of p53 were shown to induce p53-mediated anti-tumorigenic activities more than 20 years ago (Böttger et al., 1997; Kanovsky et al., 2001). Efforts to apply p53/MDM2-targeting therapeutic peptides for cancer treatment culminated in the development of a p53-derived stapled peptide, ALRN-6924. ALRN-6924 exhibits dual MDM2/MDMX inhibitory activities and has shown promise in preclinical studies and early-stage clinical trials to halt progression of cancers bearing WT p53 (Carvajal et al., 2018; Pairawan et al., 2021; Saleh et al., 2021). A recent study showed that ALRN-6924 induces inflammatory response in melanoma to alter TME and overcome tumor immune evasion, suggesting its potential utility in combination with immunotherapy (Zhou et al., 2021a). Therapeutic peptides also have potential to treat cancers with mutp53. Soragni et al. (2016) showed that a cell-penetrating peptide (CPP) derived from DNA binding domain of p53, ReACp53, inhibits mutp53 aggregation and rescues WT-like p53 functions in high-grade serous ovarian carcinomas. The concept of using targeted protein degradation (TPD) to treat

cancers with mutp53 is also being explored (Dale et al., 2021). Isobe et al. (2020) identified a small molecule, asukamycin, that serves as a "molecular glue" linking mutp53 with E3 ubiquitin ligase UBR7. Interestingly, however, that instead of degrading mutp53, treatment of asukamycin results in non-proteolytic ubiquitination and activation of mutp53 to promote cell death in TNBC cells.

Novel Therapeutic Strategies Targeting p53-MDM2 Hub

Despite the large number of candidate drugs at different stages of preclinical/clinical development, no MDM2-p53 antagonist has been approved for cancer treatment due to challenges regarding efficacy and undesired toxicity (Zanjirband and Rahgozar, 2019; Mullard, 2020). Other than identifying more drug candidates based on the similar concept, further understanding of intricate relationship between p53 and MDM2 could provide valuable insights. As mentioned previously, most MDM2-p53 antagonists were designed to disrupt N-terminal binding between MDM2 and p53 or inhibit MDM2's ubiquitin ligase activity mediated through its C-terminal RING domain. A single residue in central zinc finger domain, cysteine 305, was shown to control p53 function through interaction with RP (Lindström et al., 2007; Macias et al., 2010; Meng et al., 2015). In a mouse model ($Mdm2^{C305F}$) carrying this human cancer-associated single mutation of MDM2, it was shown that RP-MDM2-p53 pathway plays important roles in lipid metabolism and cells' response to metabolic stress (Liu et al., 2014; Liu et al., 2017b). These findings support increasing effort to develop therapies targeting zinc finger domain of MDM2, especially in the context of exploiting metabolic vulnerabilities associated with MDM2-p53 pathway. Multiple mouse models have also been utilized to suggest delicate distinctions between MDM2's ubiquitin ligase activity and ability to control p53 function. Two separate mutants of *Mdm2* (*Y487A*–Y489A in human; *I438K*–I440K in human) have been demonstrated to partially restrain p53 response to DNA damage despite losing its ability to promote p53 degradation (Tollini et al., 2014; Humpton et al., 2021). Interestingly, while $Mdm2^{Y487A}$ causes no developmental defect yet promotes p53-dependent mortality in response to sub-lethal stress in adult mice, $Mdm2^{I438K}$ leads to embryonic lethality but is tolerated when only switched on in adult mice to allow enhanced p53 response to DNA damage. These confusing discrepancies reflect a delicate balance in p53-MDM2 relationship. How to therapeutically target this equilibrium in order to control p53 dynamics could be the key to achieve balance between maximum efficacy and minimum toxicity (Purvis et al., 2012).

Another potentially useful approach is to stratify patients based on predicted response to MDM2-based therapies. It has been shown that sensitivity of cancer cells to MDM2 inhibitors could be predicted by gene signatures containing subsets of p53 target genes (Jeay et al., 2015; Ishizawa et al., 2018). Applicability of this approach remains to be seen, depending on its ability to model oscillatory relationship between p53 and MDM2. In cancer cells with mutp53, MDM2's pro-tumorigenic potential might be outweighed by its ability to suppress gain-of-function oncogenic activity of mutp53. This was recently demonstrated in pancreatic ductal adenocarcinoma, in which pharmacologic inhibition of valosin-containing protein (VCP) promotes MDM2-mediated degradation of mutp53 and cell death (Wang et al., 2021). This concept could be applied to 1) inhibit other factors disrupting MDM2-mutp53 interaction, such as HSP90 and BAG2 (Li et al., 2011b; Yue et al., 2015); or 2) tip the dynamics of MDM2-mutp53 interaction towards p53 degradation to amplify MDM2's anti-tumorigenic functions in cancers possessing mutp53 (Yang et al., 2019). Intrinsic characteristics of mutp53 could also dictate the functional consequence of p53-MDM2 interaction. A recent study demonstrated that some temperature sensitive (ts) mutp53, such as R282W and A138V, are resistant to MDM2-mediated degradation despite their ability to induce MDM2 upon reactivation. This result predicts favorable outcome of p53 reactivation in cancers possessing ts mutp53, and rationalizes including hypothermia-based treatment as part of cancer therapeutic strategy (Lu et al., 2021).

Potential Therapies Targeting Pan-MDM2 Functions

As more p53-independent functions of MDM2 are discovered, more efforts are devoted to identifying therapies targeting pan-MDM2 functions instead of MDM2-p53 interaction. A variety of small molecule inhibitors were identified to induce MDM2 auto-ubiquitination and degradation, or inhibit its interactions with non-p53 binding partners (Wang et al., 2014; Burgess et al., 2016; Singh et al., 2016; Xu et al., 2016; Nguyen et al., 2017). These inhibitors not only provide dual advantages inhibiting both p53-dependent and -independent functions of MDM2, but also demonstrate critical roles of MDM2 regulators and cofactors such as Nuclear Factor of Activated T cell (NFAT1), and X-Linked Inhibitor of Apoptosis (XIAP) (Gu et al., 2016; Gu et al., 2018; Wang et al., 2019). Inhibition of p53-independent functions of MDM2 also contributes to activities of established chemotherapeutic agents, including Adriamycin and Nilotinib (Ma et al., 2000; Zhang et al., 2014). Increasing knowledge in this field will facilitate repurposing and tailoring existing therapies towards cancers that can benefit from MDM2-targeting interventions.

TPD strategy has also been applied to develop MDM2-targeting therapies. A first-in-class MDM2 degrader using proteolysis targeting chimera (PROTAC) concept, MD-224, was shown to be highly potent in inducing MDM2 degradation and achieving durable tumor regression *in vivo* (Li et al., 2019). MD-224 consists of a modified MDM2 inhibitor conjugated with a small-molecule ligand (lenalidomide) of an E3 ligase (cereblon) degradation system. Interestingly, since MDM2 is an E3 ligase itself, MDM2-recruiting PROTAC are being developed to target itself and other pro-tumorigenic proteins to maximize p53-dependent and -independent effects in tumor suppression (Hines et al., 2019; He et al., 2021).

MDMX-Targeting Therapeutic Approaches

MDMX has emerged as a viable target for cancer therapy, both for its own ability to inhibit p53 and its role in the MDM2/MDMX complex, especially in cancers where amplification of MDMX is more prevalent than MDM2 (Gembarska et al., 2012; Burgess et al., 2016). The highly homologous yet non-identical sequence comparison between MDM2 and MDMX (>50% identical amino acid sequence in both N-terminal p53-binding and C-terminal RING domains) provides opportunities to target either MDMX specifically or the MDM2/MDMX complex. A series of molecules have been identified through MDMX-specific screens, including imidazoline derivative SJ-172550 that competes with MDMX to release functional p53 (Reed et al., 2010). SJ-172550 and other molecules subsequently identified through this approach have shown anti-tumorigenic effects and more importantly, abilities to synergize with MDM2-specific inhibitors (Reed et al., 2010; Wang et al., 2011; Wang and Yan, 2011; Karan et al., 2016). MDMX-targeting inhibitors have also been identified through indirect discoveries. Originally found to reduce proto-oncogene Survivin expression, camptothecin analogue FL118 was shown to activate p53 by promoting degradation of MDMX (Ling et al., 2014). Hsp90 inhibitor 17AAG was also found to be a potent MDMX degrader and synergize with MDM2 inhibition to activate p53 (Vaseva et al., 2011).

Specific structural features and conformational alterations upon interacting with p53 or inhibitors can inform rational drug design targeting MDM2 or MDMX. Structures of p53-MDM2 and p53-MDMX complexes revealed that their respective binding pockets are significantly different in depth and shape (Kussie et al., 1996; Popowicz et al., 2007). Distinctive conformational changes of MDM2 and MDMX upon inhibitor bindings were also identified by performing computer-aided analysis of molecular dynamics simulations (Chen et al., 2013; Chen et al., 2015). Taking advantages of these unique characteristics has led to the identification of p53 activator Inauhzin using computational structure-based screening (Zhang et al., 2012). Although Inauhzin was later found to activate p53 through inhibiting SIRT1 instead of MDMX or MDM2, similar approaches could lead to development of inhibitors distinguishing or combining MDM2-and MDMX-targeting activities.

The close structural and functional relationship between MDM2 and MDMX means that some molecules, originally identified as MDM2 inhibitors, were found to exert their activities through interfering with the MDM2/MDMX complex. The examples include MEL23 and its analogs, a number of MMRi (MDM2-MDMX RING domain inhibitors), and a pyrrolidone derivative that inhibits E3 ligase activity of MDM2/MDMX complex to activate p53 (Herman et al., 2011; Zhuang et al., 2012; Wu et al., 2015). Graves et al. (2012) instead discovered a couple of indolyl hydantoin compounds that restore p53-mediated apoptotic activity by promoting formation of dimeric complexes between MDM2 and MDMX to sequester them away from p53.

The aforementioned p53-derived stapled peptide, ALRN-6924, represents another approach to disrupt protein-protein interactions between p53 and both MDM2 and MDMX (Bernal et al., 2010; Brown et al., 2013). Interestingly, recent data from early clinical trials of ALRN-6924 showed superior toxicity profiles compared to other MDM2 inhibitors (Konopleva et al., 2020; Saleh et al., 2021). It is speculated that this observation might be attributed to ALRN-6924's dual MDM2/MDMX inhibitor status, making it a milder MDM2 inhibitor in certain tissues to minimize toxicity. It suggests that ALRN-6924, or other MDM2/MDMX dual-inhibitors, have potential as chemoprotective agents when used alongside other potent yet highly toxic chemotherapeutic drugs (Carvajal et al., 2005).

As our understanding of mechanisms surrounding MDM2 and MDMX grows (comprehensively reviewed by Klein et al.), so will our ability to design and develop cancer therapies based on disease/tissue-specific relationships between p53, MDM2 and MDMX (see reviews by Nguyen et al. and Burgess et al. for detailed overview of therapies targeting MDM2/MDMX-p53) (Burgess et al., 2016; Nguyen et al., 2017; Klein et al., 2021).

Justify the Value of ARF-Targeting Cancer Therapies

Development of ARF-targeting therapies has been handicapped by following misperceptions: 1) Linear relationship between ARF and p53: ARF and p53 are often thought to act in a linear pathway to inhibit tumorigenesis. With progress already made in developing p53-activating therapies and general difficulties in activating tumor suppressors, there is little need to devote much attention on ARF. 2) Emergence of CDK4/6 inhibitors: Recent development of selective CDK4/6 inhibitors resulted in, compared with previous generations of CDK inhibitors, lower toxicities, higher tumor-suppressive activities, and enhanced tumor immunogenicity (O'Leary et al., 2016; Goel et al., 2017). These drugs are considered magic bullets against *CDKN2A*-deficient cancers and have demonstrated promising results in preclinical/clinical settings. 3) Promise of cancer immunotherapy: Harnessing patients' own immune system to treat cancer has long been believed to be the holy grail in cancer therapy. Within the last 10 years, that belief has come to fruition with numbers of modern cancer immunotherapies, including checkpoint blockade and chimeric antigen receptor (CAR) T-cell therapies among others, dominate our attention in the fight against cancer. Cancer immunotherapy, theoretically, battles cancers in the most systematic way, bypassing needs to consider any specific aberrations in tumor-associated factors, such as tumor suppressors (Waldman et al., 2020).

A compelling argument can be made, however, to counter these misperceptions and support a strong pursuit of ARF-based cancer therapies: 1) With the number of p53-independent functions of ARF identified, there is a clear need to focus on developing therapies specifically targeting ARF-mediated pathways. ARF-based therapies have potential to synergize with p53-targeting drugs to inhibit tumorigenesis through shared tumor-suppressive mechanisms like apoptosis, autophagy and ferroptosis. 2) Despite early clinical promise, intrinsic and acquired resistance to CDK4/6 inhibitors have hindered their effectiveness (Xu et al., 2021). Mechanisms underlying resistance to CDK4/6 inhibitors, such as loss of

retinoblastoma (RB1) function, are being investigated to develop complementary or combination strategies. There is little known, however, about the role of ARF in both resistance and potential complement to CDK4/6 inhibitors. It is reasonable to believe that ARF-based therapies can provide synergistic effects with CDK4/6 inhibitors, especially in *CDKN2A*-deficient cancers. 3) Cancer immunotherapy has brought great promise, but also inevitably raised significant questions. Among challenges faced by the future of cancer immunotherapy, is understanding cancer-intrinsic factors regulating TME leading to immune evasion (Wellenstein and de Visser, 2018; Hegde and Chen, 2020). Recent studies found that genomic *CDKN2A* loss-of-function is associated with worse clinical outcome in patients treated with cancer immunotherapy in multiple cancer types (Adib et al., 2021; Gutiontov et al., 2021; Zhu et al., 2021). The reduced benefit of cancer immunotherapy can be attributed to altered tumor-immune microenvironment and compromised immune cell functions. With previous studies demonstrating ARF's ability to activate innate and adaptive immune responses within cancer cells to suppress tumorigenesis *in vivo*, ARF-targeting strategies present opportunities to augment existing cancer immunotherapies (Yetil et al., 2015; Cerqueira et al., 2020).

Development of ARF-Based Therapeutic Strategies—Therapeutic Peptides

Strategies to develop ARF-based therapies have so far been limited to gene therapy and therapeutic peptides. Adenovirus-mediated delivery of ARF had mostly been used experimentally *in vitro*, until recent studies showing its potential application using *in vivo* mouse cancer models (Saadatmandi et al., 2002; Tango et al., 2002; Cerqueira et al., 2020). This approach is expected to encounter similar obstacles faced by other gene therapies, including safety concerns and regulatory challenges, before reaching clinics (AuthorAnonymous, 2021).

In the absence of pharmaceutically proven activators, therapeutic peptides are viewed as viable alternatives to restore ARF functions. Development of therapeutic peptides in cancer therapy has seen more success and broader applications recently (Marqus et al., 2017; Xie et al., 2020). Compared to small molecules, therapeutic peptides generally have advantages of high potency, high specificity, wider range of targets and low toxicity. Recent advance and maturation of technologies for peptide synthesis, modification and delivery have helped overcome many of therapeutic peptides' shortcomings, such as poor metabolic stability, lack of oral bioavailability and high manufacturing cost (Muttenthaler et al., 2021). As peptides of 40 or less amino acids in length are regulated as small molecules for clinical applications, therapeutic peptides are uniquely positioned to fill the gap between small molecules and biologics for unmet medical needs (Rastogi et al., 2019). Peptide-based therapies also possess intrinsic characteristics, such as their propensity to cross blood-brain barrier, to make them superior drug candidates over small molecules for certain diseases (ex. central nervous system cancers) (Zhou et al., 2021b). Interference peptides designed to disrupt protein-protein interactions, like previously mentioned ALRN-6924, are relatively easy to design to achieve high target specificity and selectivity (Sorolla et al., 2020). To directly rescue or supplement for defective or deleted genes, such as tumor suppressors like ARF, peptide mimetics containing functionally significant motifs represent a new and flexible class of cancer therapeutic drugs.

A peptide containing N-terminal portion of ARF (aa 1–20) was found to bind MDM2 and inhibit p53 ubiquitination *in vitro* (Midgley et al., 2000). This observation confirmed functional significance of the N-terminal region of ARF, and synthetic peptides containing this region showed cytotoxic activity against cancer cells (Johansson et al., 2008). Although N-terminus ARF peptides display intrinsic cell-penetrating ability, potent CPPs of ARF have been generated by addition of a poly-arginine protein transduction domain (PTD) known to promote cell permeability, stability and efficacy of therapeutic peptides (Kondo et al., 2008; Allolio et al., 2018). Poly-arginine PTD has also been used to generate ARF-CPPs containing mitochondria-targeting domain (aa 38–65) to show tumor-suppressive activities in multiple cancer types (Saito et al., 2013; Saito et al., 2016). The main challenge for future development of ARF-CPPs is to achieve an acceptable balance between anti-tumor efficacy and undesired toxicity, often seen in arginine-rich CPPs to dampen confidence for their eventual clinical applications (Li et al., 2017; Lafarga et al., 2021).

Development of ARF-Based Therapeutic Strategies—Functional Antagonists Against ARF Deficiency

Another approach to develop ARF-based therapies is to identify points of synthetic lethality associated with ARF deficiency. This concept is behind the clinical success of Poly-(ADP-ribose)-polymerase (PARP) inhibitors in treatment of *BRCA1/2*-mutant cancers and has been used to identify therapeutic targets associated with defective tumor suppressors, including p53 (Gurpinar and Vousden, 2015; Patel et al., 2021). With recent advancements in cancer cohort datasets and experimental toolkits, functional proteogenomic analysis has been used to discover synthetic lethality driven by loss-of-function tumor suppressors (Xiao et al., 2020; Lei and Zhang, 2021). This strategy has been applied in cancers with high level of *CDKN2A* deficiency, but further analyses and functional validations will be needed to delineate synthetic lethality associated specifically with ARF deficiency, as most efforts to identify therapeutic opportunities associated with *CDKN2A* deficiency begin and end at p16-CDK4/6-RB pathway intervention (Oh et al., 2020; Cao et al., 2021; Satpathy et al., 2021). Using a similar approach, Zhu et al. found that breast cancer cells with *CDKN2A* mutations are more sensitive to a TTK/CLK2 inhibitor, CC-671. Whether this discovery can be attributed to ARF deficiency requires further investigation (Zhu et al., 2018).

Alternatively, pharmacogenomic screens based on concept of functional antagonism can be used to identify targetable vulnerabilities associated with dysfunctional tumor suppressors. Functionally defined or novel/diverse drug libraries are used in high-throughput screening to determine if

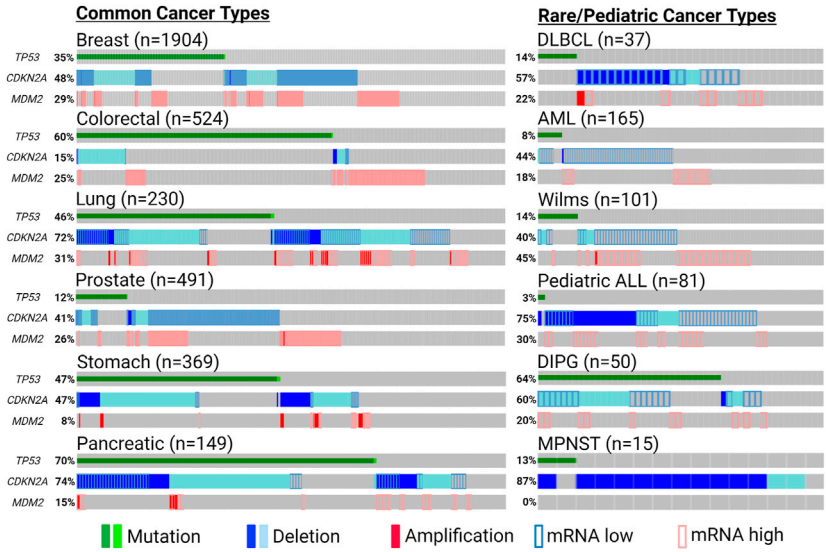

FIGURE 5 | Significant genetic alterations of p53, MDM2 and ARF in cancers. Tumor-promoting genetic events of *TP53* (non-synonymous mutation), *MDM2* (amplification or induced mRNA expression) and *CDKN2A* (deletion or reduced mRNA expression) are summarized using publicly available patient data from cBioPortal (cbioportal.org) and pediatric cBioPortal (pedcbioportal.org). Percentages shown indicate accumulated fraction of patient samples with highlighted genetic alterations. The sources of the data presented are the following: Breast—METABRIC; Colorectal/DLBCL (diffuse large b cell lymphoma)/AML (acute myeloid leukemia)—TCGA PanCancer; Lung/Prostate/Stomach/Pancreatic—TCGA Firehose; DIPG (diffuse intrinsic pontine glioma)—PNOC; Wilms/pediatric ALL (acute lymphoblastic leukemia)—TARGET; MPNST (malignant peripheral nerve sheath tumor)—MSKCC. Expression of mRNA levels (except for MPNST) are shown based on expression z-scores relative to all available diploid samples (<−0.5: mRNA low; >0.5: mRNA high). Created with BioRender.com.

the presence/absence of tumor suppressor genes in cancer cells dictates their response. This strategy could be useful to identify novel therapeutic targets or repurpose existing drugs by matching pharmacological sensitivity and genetic alterations. Bitler et al. used this approach to identify EZH2 methyltransferase as a novel target in *ARID1A*-mutated ovarian cancers, and EZH2 inhibition has since been explored as a viable therapy in other cancers with *ARID1A* mutations (Bitler et al., 2015; Alldredge and Eskander, 2017; Ferguson et al., 2021; Yamada et al., 2021). The same approach was also utilized in recent studies to identify novel therapeutic opportunities against cancers with defective RB1 tumor suppressor. Witkiewicz et al. used TNBC cells treated with CDK4/6 inhibitors and an FDA-approved drug library (1,280 compounds) to identify CHK and PLK1 inhibitors specifically antagonized by functional RB, while Gong et al. applied a limited set of drugs (36 cell-cycle inhibitors) to show that inhibition of Aurora A kinase is synthetic lethal with *RB1* mutation in a panel of diverse cancer cell lines (Witkiewicz et al., 2018; Gong et al., 2019). Interestingly, Oser et al. (2019) showed that *RB1*-deficient cancer cells are dependent on Aurora B kinase for survival by performing a synthetic lethal CRISPR/Cas9 screen in lung and breast cancer cell lines. These studies demonstrated the value of using pharmacogenomic screens to identify novel therapeutic strategies against cancers with defective tumor suppressors. It is unclear if these findings can be linked to ARF deficiency in these cancers, as ARF and p16-CDK4/6-RB function through distinctive signaling pathways. What it highlights, however, is an opportunity to fill the scientific gap by applying similar approaches with ARF-specific screens, which have not been reported in the existing literature to the best of our knowledge.

The quest to identify ARF-associated synthetic lethality could benefit from publicly curated database, such as the Biological General Repository for Interaction Datasets (BioGRID) (Oughtred et al., 2021). BioGRID compiles literature-informed data for protein/genetic/chemical interactions and CRISPR-based screens. Although no *CDKN2A*/*ARF*-specific CRISPR screens have been reported, other functional interactions revealed by the literature curation could bring interesting insight. A combinatorial CRISPR/Cas9 screen identified functional/phenotypic links between *CDKN2A* and *PTEN*, *IGF1R* and *RRM2* (Shen et al., 2017). The functional relationship between *CDKN2A* and *PTEN* has been reported, while the roles of *IGF1R* and *RRM2* might shed new light in the functions of *CDKN2A* or ARF specifically upon further studies (Carrasco et al., 2006). In another example, TRIM28 interacts with ARF to maintain chromosome integrity (Neo et al., 2015). As TRIM28 was recently shown to regulate antitumor immunity, its role in ARF-mediated immune regulation could warrant further investigation (Lin et al., 2021). With ever-growing data from multi-omics analyses being fed into databases like BioGRID, artificial intelligence-aided literature mining tool, such as CompBio (https://gtac-compbio.wustl.edu/), could facilitate our ability to extract useful information more effectively (Sapkota et al., 2021).

CONCLUSION

Countless discoveries are unquestionably to come dissecting functional interactions in and out of p53-MDM2-ARF pathway,

and many questions remain regarding how to harness their relationship to maximize clinical benefits. Does co-inactivation of p53 and ARF warrant more attention as a defective tumor-suppressive entity, for which independent investigations should be conducted instead of inferring biological meanings from their loss-of-function individually? In addition to common cancer types in which p53/MDM2/ARF alterations are prevalent, could we unveil more clinical benefits in rare and pediatric malignancies targeting this axis? Pediatric cancers have much lower mutation burdens compared to adult tumors, but most of their mutations occur in a few significant cancer driver genes, such as *TP53* and *CDKN2A* (Ma et al., 2018) (**Figure 5**). Higher significance of these pathognomonic genetic alterations could translate to better response to targeted therapies in rare and pediatric cancers (Boyd et al., 2016; Laetsch et al., 2021). Does collective status of all three genes provide additional biomarker values in helping us tailor therapeutic strategies? For example, in cancers with functional p53 and ARF in addition to MDM2 amplification, would p53-MDM2 inhibitors sensitize tumors to ferroptosis-inducing treatments? In ARF-deficient cancers with mutant p53 and MDM2 amplification, could p53/ARF-based therapeutic peptides synergize with MDM2-targeting PROTAC? With their expanding roles identified in metabolism and TME, could p53/MDM2/ARF-based interventions synergize with metabolic and immunogenic regulations? For example, mitochondrial apoptotic priming through targeting Bcl-2/Bcl-xl was recently found to significantly enhance WT p53 activity, and might have similar effects on MDM2/ARF-targeting treatments (Sánchez-Rivera et al., 2021).

To develop genetically tailored therapeutic strategies targeting cancer vulnerabilities, open access databases play critical roles in providing up-to-date and customizable resources from cancer patients, such as The Cancer Genome Atlas (TCGA) Program (https://portal.gdc.cancer.gov/) and cBioPortal for Cancer Genomics (https://www.cbioportal.org/); from diverse mouse models of human cancer, like Mouse Models of Human Cancer Database (MMHCdb: http://tumor.informatics.jax.org/mtbwi/index.do); from patient derived xenograft (PDX) models, including PDX Finder (https://www.pdxfinder.org/) and Patient-Derived Models Repository (PDMR) Database (https://pdmr.cancer.gov/); and from cancer cell lines widely available to the research community at the Cancer Dependency Map (DepMap: https://depmap.org/portal/).

Among these resources, DepMap offers an easy-to-access, genome-scale catalog to enable research in genetic and pharmacological dependencies from hundreds of cancer cell lines. Genetic dependency scores were curated from a cohort of genome-wide RNAi and CRISPR loss-of-function screens, while pharmacological dependency data were obtained from publicly sourced drug sensitivity screens. More importantly, DepMap provides built-in analytical tools to highlight genetic co-dependencies and predict novel cancer cell vulnerabilities using multi-omics gene expression profiles (Dempster et al., 2020). For example, strong co-dependencies are identified between *TP53-MDM2*/*MDMX* (negatively correlated) and *MDM2-MDMX* (positively correlated), consistent with known biological functions. Therefore, vulnerabilities associated with the p53-MDM2-ARF pathway, such as unique targets in ARF-deficient cells, could be identified for further validations. Additionally, the consolidated database for genetic information (mRNA/protein expression, copy number, mutation, methylation … etc.) from an impressive number of cancer cell lines allows identification of cell models with the desired genetic composition to conduct relevant research.

It is worth recognizing, however, limitations with these datasets. With tissue-specific tumorigenic pathways, such as p53 signaling, data to inform cancer vulnerabilities need to be considered within the proper context and cancer indications (Schneider et al., 2017). Moreover, current genetic dependency data were mostly obtained from perturbation screens against individual genes. As the dataset grows with more input from combinatorial screens targeting multiple genes simultaneously, inaccurate/incomplete connections between genetic manipulations and their physiological significance could be better avoided (Zhao et al., 2021). The same improvement could be expected as more in-depth genetic information (ex. epitranscriptomic and epigenetic modifications) are included (Kan et al., 2021). It is also important to note that, despite its increasing applications in studying cancer vulnerability, multiple recent studies have shown that CRISPR/Cas9-based technology significantly alters p53-mediated functions and signaling pathways (Haapaniemi et al., 2018; Enache et al., 2020; Jiang et al., 2021; Sinha et al., 2021). These observations indicate that this approach might compromise the critical component of unbiasedness when identifying unique cancer vulnerabilities associated with p53-surrounding networks. Despite these caveats, these resources will continue to play important roles in developing novel cancer therapies informed by genetic signatures, including the p53-MDM2-ARF complex.

The once simple triangle between p53, MDM2 and ARF has steadily grown into a complicated network merely >20 years after it was first assembled. The only thing for certain is that our fascination with this (dys)functional complex will continue for years to come, and knowledge we gain from studying its expanded network will shape the future of cancer therapy.

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Writing—original draft, formal analysis, visualization: C-PK. Supervision, funding acquisition: JW. Conceptualization, writing—review and editing: C-PK and JW.

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Olaparib Induces RPL5/RPL11-Dependent p53 Activation *via* Nucleolar Stress

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The poly (ADP-ribose) polymerase (PARP) inhibitor (PARPi) Olaparib is a widely used targeted therapy for a variety of solid tumors with homologous recombination deficiency (HRD) caused by mutation of *BRCA1/2* or other DNA repair genes. The anti-tumor activity of Olaparib has been largely attributed to its ability to inhibit PARP enzymes and block DNA single-strand break (SSB) repair, which eventually leads to the most detrimental DNA damage, double-strand breaks (DSB), in HRD cells. Although PARPi was found to induce p53-dependent cell death, the underlying molecular mechanism remains incompletely understood. Here, we report that Olaparib treatment leads to p53 stabilization and activation of its downstream target genes in a dose- and time-dependent manner. Mechanistically, Olaparib triggers nucleolar stress by inhibiting biosynthesis of the precursor of ribosomal RNAs (pre-rRNA), resulting in enhanced interaction between ribosomal proteins (RPs), RPL5 and RPL11, and MDM2. Consistently, knockdown of RPL5 and RPL11 prevents Olaparib-induced p53 activation. More importantly, Olaparib efficiently suppresses breast and colorectal cancer cell survival and proliferation through activation of p53. Altogether, our study demonstrates that Olaparib activates the nucleolar stress-RPs-p53 pathway, suggesting rRNA biogenesis as a novel target for PARPi.

Keywords: p53, ribosomal protein (RP), nucleolar (ribosomal) stress, MDM2, PARP inhibitior

INTRODUCTION

Mutation of DNA damage repair (DDR) genes is closely associated with predisposition of different types of cancer (1, 2). Both *BRCA1* and *BRCA2* are crucial to homologous recombination (HR) that is widely used by cells to repair the most detrimental DNA damage, DNA double-strand breaks (DSB). Germline mutations in *BRCA1/2* are highly prevalence in breast cancer, ovarian cancer and many other types of cancer, including lymphoma, leukemia, melanoma, prostate, pancreatic, stomach, and colorectal cancer (3–6). Poly(ADP-ribose) polymerase-1 (PARP-1) is a ubiquitous nuclear enzyme involved in multiple biological processes, such as DNA repair, cell cycle, and

apoptosis (7). It was found that the expression of PARP is significantly upregulated in various cancer cell lines and tumor tissues from patients (8–10). Recently, growing evidence has demonstrated that inhibition of PARP is a promising targeted therapy for cancer patients with deficiency in *BRCA1/2* or other DDR genes (11, 12). The first PARP inhibitor (PARPi) Olaparib has been successively used for treatment of patients with advanced solid tumors carrying a germline *BRCA1/2* mutation (13, 14). It has been shown that Olaparib achieves its therapeutic efficacy *via* several mechanisms. PARPi impairs PARP activity to mediate protein PARylation that facilitates recruitment of DNA repair components to the single-strand break (SSB) sites (7, 9). Also, PARPi was found to impede DNA replication by destabilizing replication forks, resulting in replication stress and subsequent cell death (12, 15). Furthermore, PARPi is able to induce PARP trapping, a process involving formation of a stable complex of PARPi and PARP at SSB lesions, leading to disruption of the recycle of PARP in the DDR cascade (16, 17). PARPi is believed to prevent SSB damage that may turn into DSB through aberrant DNA replication. Thus, tumor cells with HRD are particularly vulnerable to PARPi based on the genetic concept of synthetic lethality.

The tumor suppressor p53 plays an important role in DNA damage response. As a transcription factor, p53 activates the expression of a wealth of genes involved in cell cycle arrest, DNA repair, and apoptosis (18, 19). As excessive p53 activity is extreme cytotoxic, surveillance mechanisms are employed by cancer cells to inactivate p53. For instance, the E3 ubiquitin ligase MDM2 maintains a proper low level of p53 by promoting its ubiquitination and proteasomal degradation (20–24). In addition, mutations of the *TP53* gene occur in around 50% of human cancers, which not only abrogates tumor suppressive activity of p53, but also renders "gain-of-function" to drive cancer development (25). Recently, several studies have indicated that p53 activity may enhance tumor response to PARPi, as these agents can activate p53 to trigger apoptosis and ferroptosis (26, 27) or repress RAD51-mediated HR repair (28). Additionally, we have recently demonstrated that long noncoding RNA RMRP is an inhibitor of p53 in response to PARPi treatment, while targeting RMRP significantly bolsters p53 activation and enhances tumor sensitivity to PARPi (29, 30). However, the mechanisms underlying how PARPi induces p53 activation are still incompletely understood.

In this study, we reveal that Olaparib treatment induces p53 stabilization and activation in time- and dose-dependent manner. Interestingly, Olaparib represses ribosomal RNA (rRNA) biosynthesis, consequently leading to nucleolar stress (or ribosomal stress). It has been well-documented that perturbation of ribosome biogenesis promotes translocation of ribosomal proteins (RPs) from the nucleolus to the nucleus where they can associate with MDM2 and inhibit MDM2-mediated p53 degradation (31–34). Herein, we elaborate that Olaparib treatment enhances interaction between RPL5/RPL11 and MDM2, whereas knockdown of RPL5 or RPL11 impairs Olaparib-induced p53 activation. Consistently, HCT116 $^{p53+/+}$ cells exhibit higher sensitivity to Olaparib than HCT116 $^{p53-/-}$ cells. Therefore, our study unveils rRNA biogenesis as an alternative target of PARPi, and demonstrates a novel action mode of PARPi *via* the nucleolar stress-RPs-p53 axis.

MATERIALS AND METHODS

Cell Culture and Olaparib Treatment

Human Colorectal Cancer cell lines HCT116$^{p53+/+}$, HCT116$^{p53-/-}$ and Breast cancer cell line Cal51 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 0.1 mg/ml streptomycin and maintained at 37°C in a 5% CO_2 humidified atmosphere. The cells were treated with different doses of Olaparib (MCE, Shanghai, China) and harvested at indicated time courses or dose for the future experiments.

SiRNAs and Antibodies

The siRNA sequences were used in this paper as below, siNC: UUCUCCGAACGUGUCACGU, siRPL5: 5'-GGAGGAGAUG UAUAAGAAATT-3', siRPL11: 5'-GGAACUUCGCAUCCG CAAATT-3'. All siRNAs were synthesized by Genepharma company (Shanghai, China). The anti-p53 (Catalog sc-126, Santa Cruz Biotechnology), anti-MDM2 (Catalog# M4308, Sigma), anti-p21 (Catalog#2947, Cell Signaling Technology), anti-RPL5 (Catalog ab86863, Abcam), anti-RPL11 (Catalog ab79352, Abcam), anti-GAPDH (Catalog 60004-1-Ig, Proteintech), anti-β-actin (Catalog ARG62346, Proteintech), anti-α-tubulin (Catalog 66031-1-Ig, Proteintech) were commercially purchased.

Immunoblot and Co-Immunoprecipitation Assays

Cells were lysed with lysis buffer consisting of 50 mM Tris/HCl (pH7.5), 0.5% Nonidet P-40 (NP-40), 1 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF),10 μM pepstatin A and 1 μg/ml leupeptin. Equal amounts 60 μg of clear cell lysates were used for immunoblot analysis. Co-IP assays were conducted using antibodies as indicated in the figure legends. In brief, 1 mg of total proteins were incubated with the indicated antibody at 4°C for overnight, and then Protein A or G beads were added and the mixture was left to incubate at 4°C for additional 2 h. At last, the beads were washed five times with lysis buffer. Bound proteins were detected by IB with antibodies as indicated in the figure legends.

Immunofluorescence Assay

Cells were fixed with methanol in −20°C for overnight. The fixed cells were washed by PBS and blocked with 8% BSA in PBS for 1 h followed by incubation with the anti-Flag antibody in 2% BSA in 4°C for overnight. The cells were then washed and incubated with the secondary antibody and DAPI.

Reverse Transcription and Quantitative RT-PCR Analyses

Total RNA was isolated from cells using RNAiso Plus (Takara, Dalian, Liaoning, China) according to the manufacturer's protocol. Total RNAs of 1μg were used as templates for reverse transcription reaction using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, Liaoning, China). Quantitative RT-PCR was conducted using ChamQ SYBR qPCR Master Mix (Novazyme, Nanjing, China) according to the manufacturer's protocol. The following primers were used as below: Actin-F: 5'-CATGTACGTTGCTATCCAGGC-3', Actin-R: 5'-CTCCTTAATGTCACGCACGAT-3', Puma-F: 5'-GACCTCAACGCACAGTACGAG-3', Puma-R: 5'-AGGAGTCCCATGATGAGATTGT-3', BTG2-F: 5'-ACGGGAAGGGAACCGACAT-3', BTG2-R: 5'-CAGTGGTGTTTGTAGTGCTCTG-3', MDM2-F: 5'-GAATCATCGGACTCAGGTACATC-3', MDM2-R: 5'-TCTGTCTCACTAATTGCTCTCCT-3', BAX-F: 5'-CCCGAGAGGTCTTTTTCCGAG-3', BAX-R: 5'-CCAGCCCATGATGGTTCTGAT-3', p21-F: 5'-CTGGACTGTTTTCTCTCGGCTC-3', p21-R: 5'-TGTATATTCAGCATTGTGGGAGGA-3', 112-bp-F: 5'-TGAGAAGACGGTCGAACTTG-3', 112-bp-R: 5'-TCCGGGCTCCGTTAATGATC-3', 96-bp-F: 5'-GGCCATACCACCCTGAACGC-3', 96-bp-R: 5'-CAGCACCCGTATTCCCAGG-3'. The 112-bp pre-rRNA fragment encompasses 5'-external transcribed sequence (5'-ETS) and 18S rRNA. The 96-bp pre-rRNA fragment is from 18S rRNA to internal transcribed sequence-1 (ITS-1) (35). The primers were sythesized by GENEWIZ (Suzhou, China).

RNA Interference

RNA interference-mediated knockdown of endogenous RPL5 and RPL11 were performed as described previously (33). These siRNA duplexes were introduced into cells using Hieff Trans liposomal transfection reagent (Yeasen, Shanghai, China) according to the manufacturer's protocol. The transfected cells were treated with or without 10 μM of Olaparib for 24 h before harvesting. Cells were harvested at 48 h of post transfection for immunoblot.

Cell Viability assay

To detect the proliferation of cells, the Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Japan) was used according to the manufacturer's instructions. In briefly, 3000 cells per well were seeded in 96-well culture plates with Olaparib. Cell viability was determined by WST-8 at a final concentration of 10% to each well, and the absorbance of the samples was measured at 450 nm using a Microplate Reader at 24h as indicated.

Cell Apoptosis Analysis Using Flow Cytometry

Apoptosis was analyzed by flow cytometry using an Annexin PE-V apoptosis detection kit (Yeasen, Shanghai, China) according to the manufacturer's instructions. Cells were treated with Olaparib as indicated in the figure legends. Briefly, the cells were washed with cold PBS twice, and then resuspended with 1x Binding buffer and stained with Annexin V/PI reagent in the dark for 15 min. The cells were immediately analyzed by flow cytometry after terminating the staining reaction.

Statistics

Statistical analyses were performed using GraphPad Prism 8 software. Data of experiments are expressed as mean ± standard deviation (SD) of at least three independent experiments. The Student's *t* test or one-way analysis of variance was performed to evaluate the differences between two groups or more than two groups. $p < 0.05$ was considered as statistical significance, and the asterisks represent significance in the following way: *$p < 0.05$, **$p < 0.01$, and ***$p<0.001$.

RESULTS

The PARP Inhibitor Olaparib Activates the p53 Pathway

Our study recently reported that a very low dose of Olaparib induces p53 expression upon RMRP depletion in colorectal cancer cells (29). We are curious to know whether Olaparib regulates p53 expression and activity in the normal culture condition. To test this possibility, we first evaluated the expression of p53 in response to titrated doses of Olaparib, and found that p53 is upregulated in both HCT116 $^{p53+/+}$ colorectal cancer cells and Cal51 breast cancer cells in a dose-dependent fashion (**Figures 1A, C**). Accordingly, the expression of p53 target genes, including p21, BAX, BTG2, MDM2 and PUMA, was also dose-dependently elevated by Olaparib treatment (**Figures 1A–D**). The minimal dose of Olaparib necessary for competent activation of p53 was about five micromolars in both cell lines as evidenced by the induction of p21 expression (**Figures 1A, C**). In addition, another PARP inhibitor, Niraparib, also induced the expression of p53 and p21 (**Figure S1A**). Next, to further explore the time kinetics of Olaparib-induced p53 activation, we examined p53 and its target gene expression at different time points of 10 μM Olaparib treatment (**Figures 1E-H**). The initial induction of p53 and p21 was first observed at 8 h post-treatment in HCT116 $^{p53+/+}$ cells and 4 h post-treatment in Cal51 cells (**Figures 1E, G**). Moreover, the expression of multiple p53 target genes were upregulated in HCT116 $^{p53+/+}$ and Cal51 cell lines in a time-dependent fashion (**Figures 1F, H**). The activation of p53 might not engage other oncogenic signals, as Olaparib could induce p53 in normal ovarian surface epithelial cells, IOSE-80 (**Figure S1B**). Interestingly, we found that Olaparib induces the expression of TIGAR and DRAM1 (**Figures S1C, D**), two p53 target genes critical for glucose metabolism and autophagy, respectively. Therefore, these results indicate that Olaparib treatment leads to activation of the p53 signaling pathway in a dose- and time-dependent manner in colorectal and breast cancer cells.

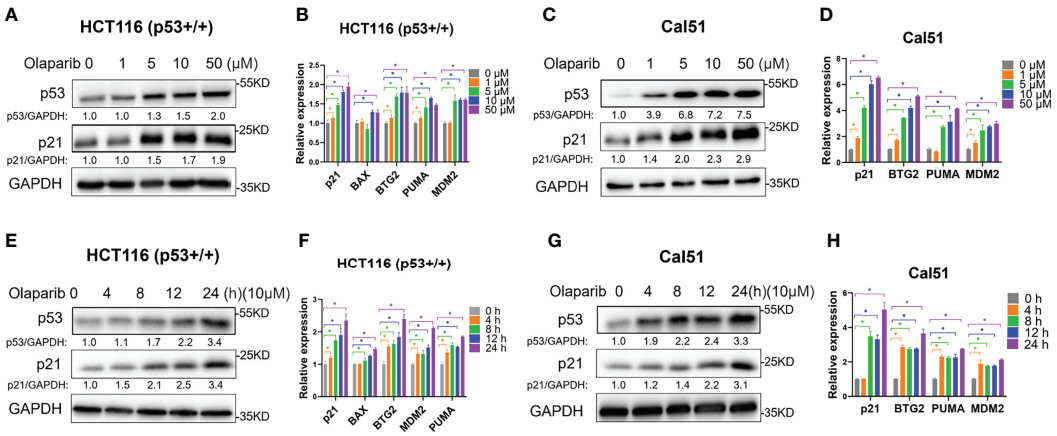

FIGURE 1 | Olaparib treatment activates the expression of p53 and its target genes. **(A, B)** Olaparib treatment elevates the protein **(A)** and mRNA **(B)** levels of p53 and its target genes in a dose-dependent manner in HCT116 $^{p53+/+}$ cells. **(C, D)** The experiments were performed as the same as **(A, B)** except that Cal51 cells were used. **(E, F)** Olaparib treatment elevates the protein **(E)** and mRNA **(F)** levels of p53 and its target genes in a time-dependent manner in HCT116 $^{p53+/+}$ cells. Cells were treated with 10 μM of Olaparib and harvested for IB at different time points as indicated. **(G, H)** The experiments were performed as the same as **(E, F)** except that Cal51 cells were used. *p < 0.05.

Olaparib Induces p53 Stabilization

It was shown by our and other groups that activation of p53 is largely due to p53 protein stabilization (18, 29, 34). In keeping with this notion, we also wondered if Olaparib affects p53 protein stability, and therefore performed the cycloheximide-chase analysis of p53 protein half-life. As shown in **Figures 2A, B**, Olaparib treatment indeed led to p53 stabilization as indicated by the prolonged protein half-life in HCT116 $^{p53+/+}$ cells. Consistently, the p53 half-life was also significantly extended upon Olaparib treatment of Cal51 cells (**Figures 2C, D**). These results reveal that Olaparib can stabilize p53 in different types of cancer cells.

Olaparib Represses Ribosomal RNA Biogenesis

Although several studies reported that PARPi may induce p53 activation (26–29), the molecular basis remains unclear. It was previously shown that small nucleolar RNA (snoRNA)-mediated PARP-1 activation contributes to ribosome biogenesis (36). Since we and others have established that impairment of ribosome biogenesis leads to nucleolar stress and consequent p53 activation (31–34), we therefore sought to determine if Olaparib triggers nucleolar stress. We first examined whether Olaparib inhibits biosynthesis of rRNAs, the critical component of the ribosome, by directly comparing

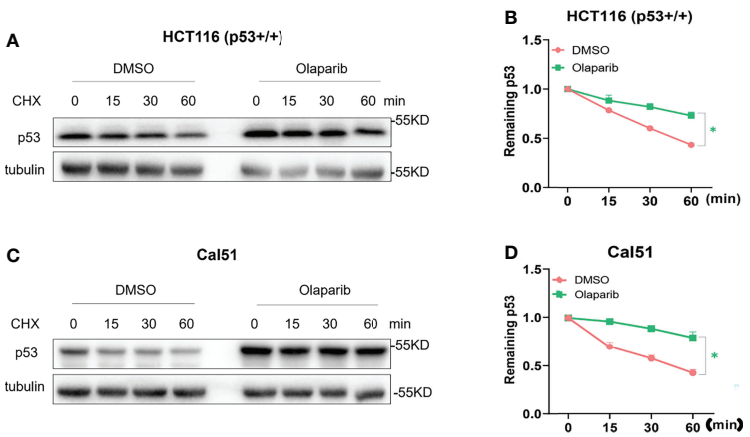

FIGURE 2 | Olaparib treatment promotes p53 stabilization. **(A, B)** HCT116 $^{p53+/+}$ cells were treated with 10 μM Olaparib for 12 h, and cycloheximide was added to the cultures at different time points before harvest as indicated. Cells were harvested and subject to the IB assay **(A)**, and p53 expression was quantified as shown in the panel **(B)**. **(C, D)** The experiment was performed as the same as **(A, B)** except that Cal51 cells were used. The cycloheximide-chase assay was performed in triplicate, *p < 0.05.

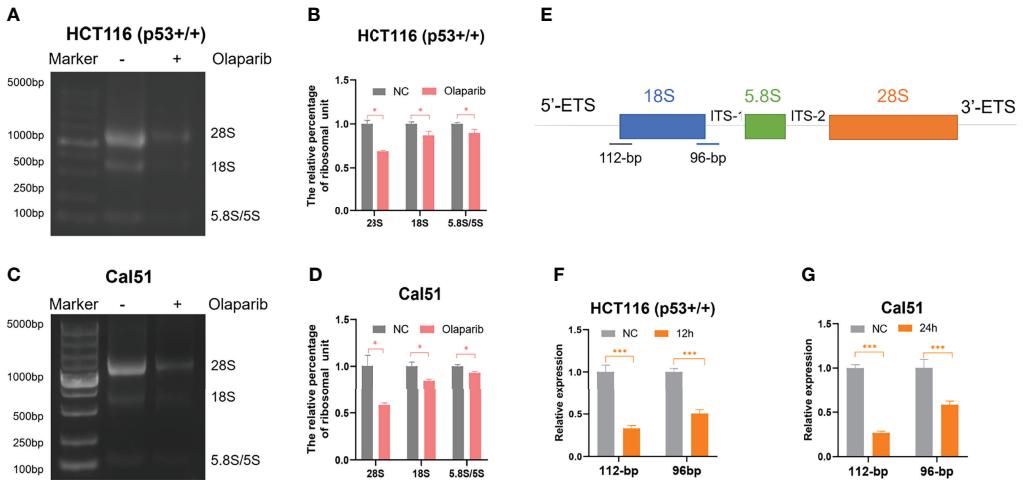

FIGURE 3 | Olaparib treatment inhibits pre-rRNA biosynthesis. **(A, B)** HCT116 $^{p53+/+}$ cells were treated with or without 10 μM Olaparib for 12 h. The expression of 28S, 18S, and 5.8S rRNA was analyzed by agarose gel electrophoresis **(A)** and quantified as show in the panel **(B)**. **(C, D)** The experiment was performed as the same as **(A, B)** except that Cal51 cells were used. **(E)** A schematic illustration of the pre-rRNAs structure and the fragments amplified by RT-qPCR. **(F, G)** HCT116 $^{p53+/+}$ **(F)** and Cal51 **(G)** cells were treated with or without 10 μM Olaparib for 24h, and then harvested and subject to RT-qPCR by amplifying a 112-bp fragment through 5'-ETS and 18S rRNA and the other 96-bp fragment encompassing 18S rRNA and ITS-1 as indicated. *$p < 0.05$ and ***$p < 0.001$.

28S, 18S, and 5.8S/5S rRNAs through gel electrophoresis (**Figures 3A–D**). The result showed that Olaparib treatment of both HCT116 $^{p53+/+}$ (**Figures 3A, B**) and Cal51 (**Figures 3C, D**) cell lines significantly reduces the levels of rRNAs. Importantly, our data also demonstrated that Olaparib suppresses the production of 28S, 18S, and 5.8S/5S rRNAs in a dose- and time-dependent manner in both cell lines (**Figures S2A–D**). p53 was not required for this process, as Olaparib could still inhibit rRNA production in HCT116 $^{p53-/-}$ cells (**Figure S3**). Biosynthesis of nucleolar rRNAs involves rDNA transcription and pre-rRNA processing into three subtypes of mature rRNAs, including 28S, 18S, and 5.8S rRNAs (32). To elucidate if Olaparib regulates the level of pre-rRNA or not, we performed RT-qPCR by amplifying two fragments encompassing 5'-ETS and 18S rRNA, or 18S rRNA and ITS-1 (35) as indicated in **Figure 3E**. Remarkably, the result clearly showed that Olaparib dramatically reduces the pre-rRNA levels in both cancer cell lines (**Figures 3F, G**). Together, these results suggest that Olaparib treatment can inhibit pre-RNA synthesis to trigger nucleolar stress.

Olaparib Induces RPL5/RPL11-Dependent p53 Activation

Since nucleolar stress elicits the release of RPL5 and RPL11 into the nucleus where they repress MDM2-induced p53 degradation by directly binding to MDM2 (31–34, 37), we attempted to elaborate if Olaparib also provokes the RPs-MDM2-p53 cascade by inducing nucleolar stress. First, we conducted the immunofluorescence assay and found that Flag-L5 and Flag-L11 are mainly localized in the nucleolus (for pre-ribosome assembly) and the cytoplasm (for protein translation) in untreated cells, while the nucleolar localization of RPL5 and RPL11 are disrupted in response to Olaparib treatment (**Figures 4A, B**). Next, by performing a set of co-IP assays using the anti-MDM2 antibody, we showed that Olaparib treatment indeed enhances the interaction between MDM2 and both RPs, respectively (**Figures 4C, D**). Furthermore, we wondered if RPL5 and RPL11 are required for p53 activation in response to Olaparib-induced nucleolar stress. As shown in **Figure 5**, depletion of RPL5 with siRNA markedly inhibited Olaparib activation of p53 in HCT116 $^{p53+/+}$ and Cal51 cells (**Figures 5A, B**). Consistently, the same effect was achieved by knocking down RPL11 in both cell lines (**Figures 5C, D**). Taken together, these results demonstrate that Olaparib activation of p53 requires RPL5 and RPL11 binding to MDM2.

Olaparib Suppresses Cancer Cell Growth Partially Dependent on p53

Given the action of Olaparib to trigger the nucleolar stress-p53 pathway, we determined if the p53 status is correlated with cytotoxic effect of Olaparib by using the wild-type p53-harboring HCT116 $^{p53+/+}$ and Cal51 cell lines and the p53-null HCT116 $^{p53-/-}$ cell line. The cell viability assay was performed to show that Olaparib significantly represses HCT116 $^{p53+/+}$ and Cal51 cell growth in a dose-dependent manner (**Figures 6A, B**), which is in line with the former results (**Figures 1A–D**). It was noted that as low as 5 μM Olaparib is able to markedly suppress Cal51 and HCT116 $^{p53+/+}$ cell proliferation (**Figures 6A, B**), probably because p53 can be fully activated at this dosage (**Figures 1A, C**). More importantly, the p53-depleted Cal51 and HCT116 $^{p53-/-}$ cell lines exhibited much lower sensitivity to Olaparib compared to their isogenic counterparts (**Figures 6A, B**). Moreover, we also examined the effect of Olaparib on

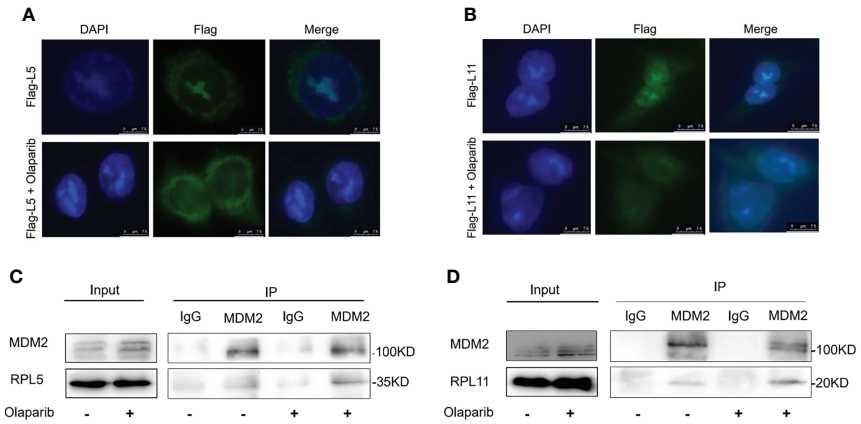

FIGURE 4 | Olaparib treatment enhances the interaction of MDM2 with RPL5 and RPL11. **(A)** HCT116 $^{p53+/+}$ cells were transfected with Flag-L5 and treated with or without 20 μM Olaparib, and subject to the immunofluorescence assay. **(B)** The experiment was performed as the same as **(A)**, except that Flag-L11 was introduced into cells. **(C)** Cal51 cells were treated with or without 20 μM Olaparib for 24 h and harvested for co-IP-IB analysis. RPL5 was co-immunoprecipitated with MDM2 using an anti-MDM2 antibody. **(D)** The experiment was performed as the same as **(C)**, except that RPL11 was co-immunoprecipitated with MDM2 using an anti-MDM2 antibody.

apoptosis in these cell lines. Consistently, Olaparib drastically induced apoptosis of HCT116 $^{p53+/+}$ and Cal51 cells, while had a moderate effect on the induction of apoptosis in HCT116 $^{p53-/-}$ cells (**Figures 6C–F**). Finally, we showed that Olaparib triggers cell cycle arrest at G2 phase in HCT116 $^{p53+/+}$ (**Figures S4A, B**) and Cal51 cells (**Figures S4C, D**), which is in line with the former results that Olaparib induces p21 expression (**Figure 1**). Therefore, our results demonstrate that Olaparib inhibits cell proliferation and promotes apoptosis partially dependent on p53.

DISCUSSION

The tumor suppressor p53 plays a vital role in preventing tumorigenesis by regulating the expression of a myriad of genes involved in DNA damage response and apoptosis. Inactivation of p53 usually leads to cancer development and therapeutic resistance (18, 19, 29, 38, 39). In this study, we showed that Olaparib treatment promotes p53 protein stabilization and thus upregulates p53 target gene expression in a dose- and time-dependent manner (**Figures 1**, **2**). Mechanistically, Olaparib was

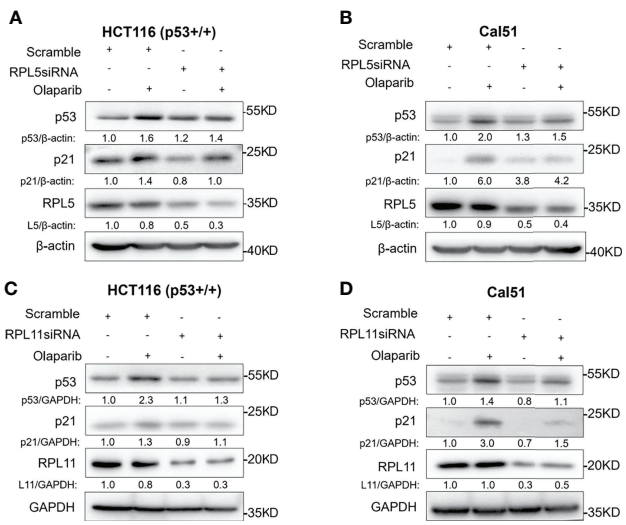

FIGURE 5 | Olaparib-induced p53 activation requires RPL5 and RPL11. **(A, B)** HCT116 $^{p53+/+}$ **(A)** and Cal51 **(B)** cells were transfected with control or RPL5 siRNA for 24 h, and then treated with or without 10 μM Olaparib for another 24 h before harvest for IB analysis. **(C, D)** The experiments were performed as the same as **(A, B)** except that RPL11 siRNA was used.

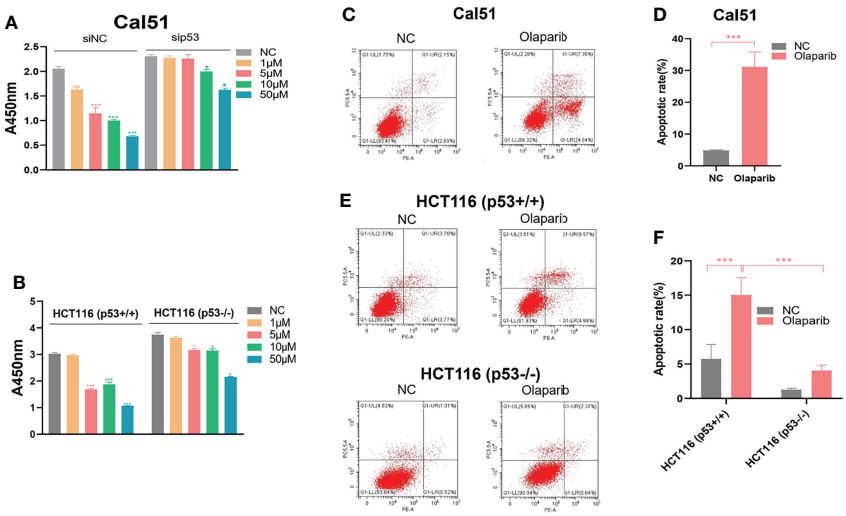

FIGURE 6 | Olaparib suppresses growth and prompts apoptosis of cancer cells. **(A, B)** Cell viability assay was performed to assess the growth of Cal51, p53-depleted Cal51, HCT116 $^{p53+/+}$ and HCT116 $^{p53-/-}$ cells upon Olaparib treatment as indicated. **(C, D)** Apoptosis of Cal51 cells treated with or without Olaparib was assessed by flow cytometry. **(E, F)** Apoptosis of HCT116 $^{p53+/+}$ and HCT116 $^{p53-/-}$ cells treated with or without Olaparib was assessed by flow cytometry. *p < 0.05 and ***p<0.001.

found to trigger nucleolar stress by inhibiting pre-rRNA biosynthesis (**Figure 3**), consequently leading to enhanced interaction between RPL5/RPL11 and MDM2 (**Figure 4**). Conversely, knockdown of RPL5 or RPL11 by siRNAs markedly impaired Olaparib-induced p53 activation (**Figure 5**). More importantly, Olaparib suppressed breast and colorectal cancer cell survival and proliferation partially through activation of p53 (**Figure 6**). Taken together, our study uncovers an unexplored therapeutic action of PARPi by activating the p53 pathway. MDM2 binds to and promotes degradation of p53 under the normal condition, while PARPi induces nucleolar stress to release RPL5 and RPL11 into the nucleoplasm, enhancing the interactions between the RPs and MDM2 and, consequently leading to p53 stabilization and activation (**Figure 7**).

PARPi has been widely used in the treatment of tumors with *BRCA1/2* mutation or HRD (13, 14). The *TP53* gene status was also reported to be associated with tumor sensitivity to PARPi. Several studies suggest that *TP53* mutation predicts enhanced cytotoxicity of PARPi, because inactivation of p53 accelerates cell cycle progression and impairs the DDR pathways, thus accumulating unrepaired DNA damage (40). Alternatively, missense mutant p53 can associate with PARP-1 to promote aberrant repair of the damaged DNA caused by the alkylating agent, which may create a strong tumor dependency on PARP-1

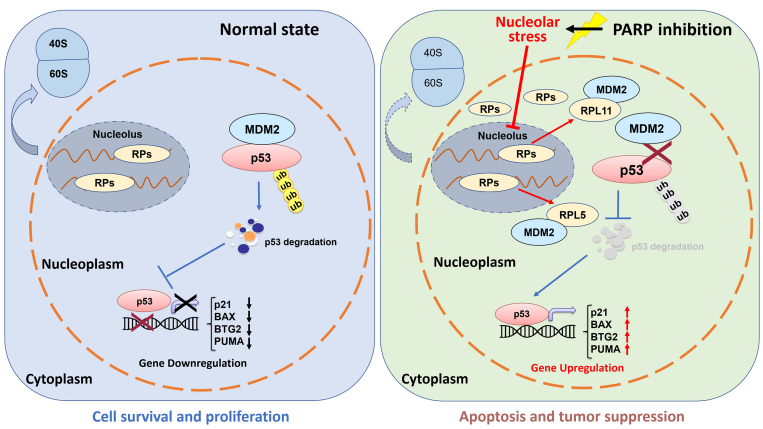

FIGURE 7 | Working model of Olaparib activation of p53 *via* nucleolar stress. Under the normal condition, RPs and rRNAs work together for pre-ribosome assembly in the nucleolus, while MDM2 binds to p53 and maintains a relatively low level of p53 in cells (left panel). Olaparib treatment inhibits pre-rRNA biosynthesis, thus leading to nucleolar stress. Many RPs, such as RPL5 and RPL11, are released to the nucleoplasm to interact with MDM2 resulting in p53 stabilization and activation, and consequent p53 target gene upregulation.

(41, 42). By contrast, PARPi was also shown to repress wild-type p53-harbroing tumors by inducing p53-dependent apoptosis or ferroptosis (26, 27, 43). In addition, we have recently reported that depletion of RMRP elicits full activation of p53 under Olaparib treatment, leading to tumor sensitization to PARPi-associated therapies (29). These seemingly contradictory findings indicate that p53-dependent DDR and cell death play distinct roles in PARPi treatment of cancer. However, it is not very clear how PARPi activates p53. We also showed that Olaparib-induced p53 activation is coincident with the elevation of phosphorylation of γ-H2AX, a maker for DNA damage (**Figures S5A, B**). One possible mechanism is that PARPi-caused replication stress and the consequent DNA damage stress may induce p53 activation, though the detailed mechanism is yet to be investigated. In this study, nevertheless, we clearly demonstrate that PARPi activation of p53 involves perturbation of ribosome biogenesis and interaction of the ribosome-free RPs with MDM2, which provides the first mechanistic insight into how these agents activate the p53 pathway. Remarkably, our study suggests that PARP and the nucleolus may be the dual targets for PARPi, and that these agents could be used in tumors with HRD and/or active ribosome biogenesis.

It has long been noticed that the nucleoli are morphologically altered in transformed or cancer cells (44, 45), because these cells often sustain a high rate of ribosome biogenesis to fulfill the requirement for their own rapid growth and propagation. Thus, interference with rRNA and RP synthesis or ribosome assembly, which causes nucleolar stress and consequent p53 activation, has become a promising anti-cancer strategy (31, 32). It was found that a low dose of Actinomycin D (<10 nM) selectively inhibits rDNA transcription, although it may also lead to DNA damage stress at a higher dose. In addition, several DNA damage-based therapies, such as 5-Fluorouracil, Cisplatin, Doxorubicin, and UV or γ-irradiation, are able to induce nucleolar stress by repressing rDNA transcription or rRNA processing. Some RPs, such as RPL37, were reported to undergo degradation in response to the genotoxic insults, Cisplatin and UV light. Moreover, mycophenolic acid, an immunosuppressant drug, was found to disturb the nucleolar architecture and impair rRNA synthesis. Recently, several small molecules with anti-cancer activity have been developed to selectively inhibit rRNA production. CX-3543 was identified as an inhibitor of G-quadruplexes that are crucial to transcription of GC-rich rDNAs (46). Another nucleolar stress-inducing agent CX-5461 impedes recruitment of SL1, a critical component of the RNA Pol I initiation complex, on the rDNA promoter (47). BMH-21 associates with GC-rich rDNA genes to suppress RNA Pol I function, as well as promotes proteasomal degradation of the RNA Pol I catalytic subunit RPA194 (48). In our attempt to elucidate the molecular basis behind PARPi activation of p53, we showed that Olaparib treatment markedly inhibits production of pre-rRNAs, leading to reduced levels of the 28S, 18S, and 5.8S rRNAs (**Figure 3**), which is in line with a previous study showing that PARylation of the RNA helicase DDX21 by PARP-1 facilitates rDNA transcription (36). As expected, we further demonstrated that the inhibition of rRNA biosynthesis leads to nucleolar stress in which ribosome free-RPL5 and -RPL11 interact with MDM2 to stabilize p53 (**Figures 4**, **5**). Nevertheless, a few questions are also raised based on our findings. Whether or not other RPs and ribosome-related proteins, such as RPL23 (49, 50), RPS14 (33), and SBDS (34), are involved in PARPi-induced p53 activation remains to be understood. Since the *TP53* gene is mutated in around 50% of human cancers, it is worthwhile to investigate if PARPi-triggered nucleolar stress regulates mutant p53 signaling. Given that ribosome-free RPs also interact with TAp73 (51) and c-Myc (52), it is intriguing to test if PARPi modulates these signaling pathways independently of p53 *via* eliciting nucleolar stress.

CONCLUSION

PARPi have been widely used for treatment of tumors harboring *BRCA1/2* mutation or with HRD as a synthetic lethal agent. Recent studies revealed that PARPi can induce p53-dependent cell death that contributes to the anti-cancer effect of this agent. However, the molecular mechanism underlying how PARPi activates the p53 pathway is elusive. In this study, we demonstrate for the first time that Olaparib suppresses rRNA biosynthesis, thus eliciting the nucleolar stress-RPs-p53 axis and consequent cancer cell apoptosis. Our study also suggests that rRNA biogenesis could be an alternative target for PARPi, which is worthwhile for clinical test in future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further rational inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The study was approved by both the Ethics Committee of Xinxiang Medical University and Fudan University Shanghai Cancer Center.

AUTHOR CONTRIBUTIONS

TH conducted and analyzed part of the experiments and provided critical reagents and materials. JT conducted and analyzed most of the experiments. MW and YG performed part of IB analysis. BG, JC, and YL provided important instructions and helped to analyze the data. TH, QH and XZ conceived, designed and supervised the study, and analyzed the data. TH, JT and XZ drafted the manuscript. QH and XZ edited the manuscript. All authors contributed to the article and approved the submitted version. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.821366/full#supplementary-material

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