HUMAN TUMOR-DERIVED P53 MUTANTS: A GROWING FAMILY OF ONCOPROTEINS

EDITED BY: Ygal Haupt and Giovanni Blandino
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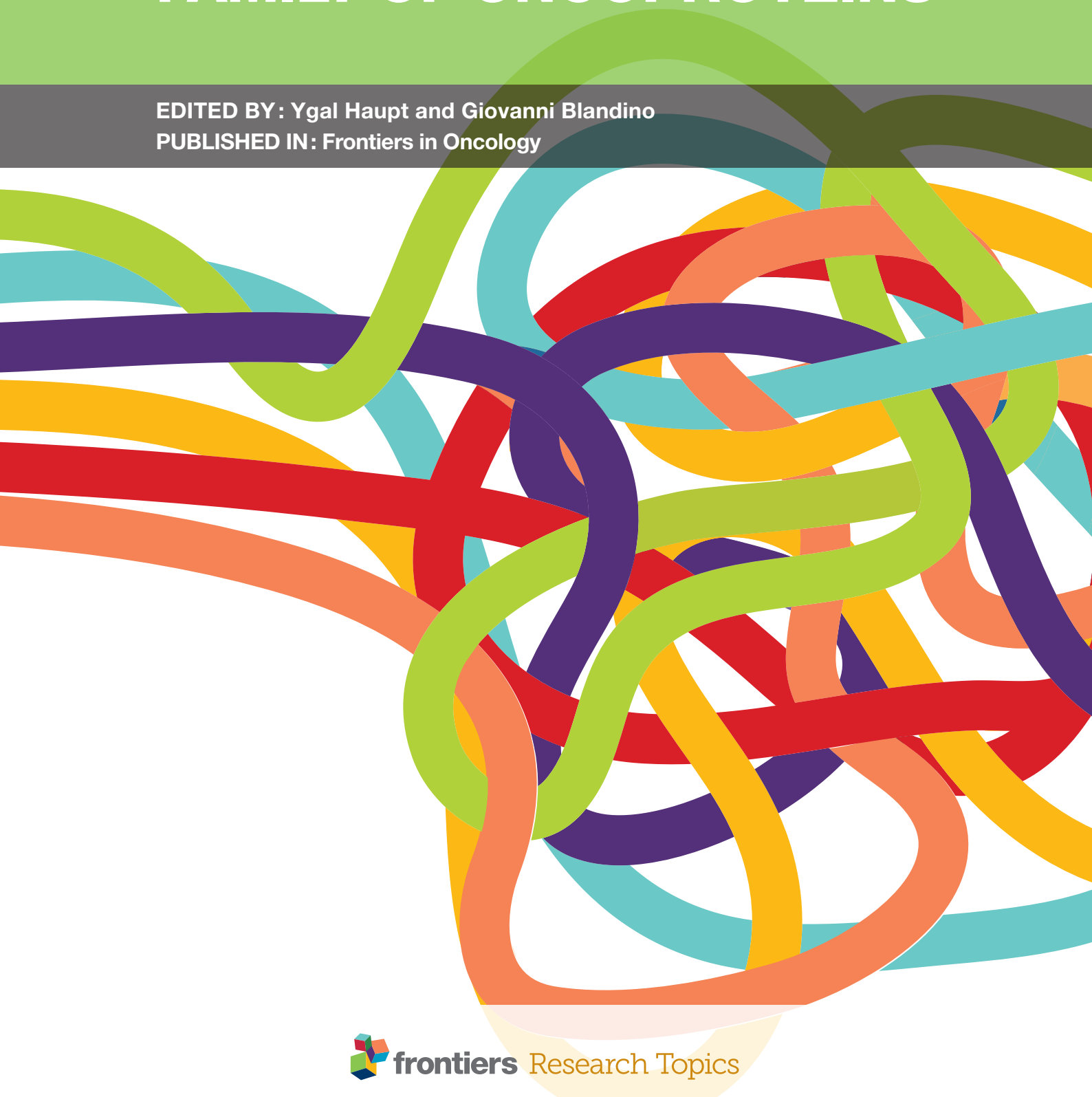

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HUMAN TUMOR-DERIVED P53 MUTANTS: A GROWING FAMILY OF ONCOPROTEINS

Topic Editors:
Ygal Haupt, Peter MacCallum Cancer Centre & The University of Melbourne & Monash University, Australia
Giovanni Blandino, Italian National Cancer Institute Regina Elena, Italy & McMaster University, Canada

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Editorial: Human Tumor-Derived p53 Mutants: A Growing Family of Oncoproteins

Ygal Haupt[1,2,3,4]* *and Giovanni Blandino*[5,6]

[1] Tumour Suppression Laboratory, Peter MacCallum Cancer Centre, Melbourne, VIC, Australia, [2] Sir Peter MacCallum Department of Oncology, The University of Melbourne, Parkville, VIC, Australia, [3] Sir Peter MacCallum Department of Pathology, The University of Melbourne, Parkville, VIC, Australia, [4] Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC, Australia, [5] Oncogenomic and Epigenetic Unit, Italian National Cancer Institute Regina Elena, Rome, Italy, [6] Department of Oncology, McMaster University, Hamilton, ON, Canada

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The Editorial on the Research Topic

Human Tumor-Derived p53 Mutants: A Growing Family of Oncoproteins

Mutations in the tumor suppressor *p53* gene are collectively the most common event in human cancers. These do not merely reflect a loss of the tumor suppressive function of wild type (wt) p53 but are also selected during tumorigenesis for their acquired gain-of-function (GOF), together contributing to multiple hallmarks of cancer. Over 30 years of extensive study into wt p53 provided a wealth of information about its regulation, functions, and contribution to cancer prevention. Albeit, with a significant delay, the interest in mutant p53 has been growing fast over the past decade with the realization that most cancer patients present tumors with mutant p53, and these particularly manifest in aggressive and metastatic diseases. The growing understanding of mutant p53 exposes attractive therapeutic opportunities with wide clinical applications, which coincidently raise many challenging questions concerning associated complexities. In this research topic, we assembled 12 reviews exposing some critical issues and discussing prospect development in this field.

One of the most commonly used mouse models for cancer is the p53 knockout mouse. However, this model of p53 deficiency does not represent the majority of human cancers. A major leap in the understanding of mutant p53 regulation and GOF was derived from mouse genetics (1, 2). The group of Lozano, which led the mouse models for mutant p53, highlighted the GOF learned from the comparison between p53 deficient mice and mutant p53 knock-in mice, primarily the contribution of mutant p53 to tumor metastasis (Kim et al.). The Lozano group also emphasized the biological and biochemical differences between different mutants, even between different substitutions of the same amino acid, such as p53R172H versus p53R172P. Hence, not all p53 mutants are equal. While the abovementioned mouse models for inherited p53 mutations (the Li–Fraumeni model), this represents a small fraction of p53 mutations in human cancers. This key point was discussed by the Lozano group highlighting the limitations of the current mouse models for sporadic p53 mutations in cancer. They discuss the problems with the current conditional mutant p53 models in which all cells are heterozygous for p53 from conception and hence do not faithfully mimic the role of mutant p53 in sporadic tumor development. These models lack the challenging context of the cells with wt p53 that normally comprise the tumor microenvironment and its inherent immune cells. There is a clear need for more sophisticated mouse models to better define the distinct roles of mutant p53 in these compartments. The Del Sal group (Walerych et al.) discussed the difference between the mouse and human mutant p53 exemplified by the p53R249S mutation, which exhibits GOF in human cells, but this had not been recapitulated in the relevant mouse model. A comprehensive list summarizing

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Carlotta Giorgi,
University of Ferrara, Italy

***Correspondence:**
Ygal Haupt
ygal.haupt@petermac.org

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studies from the last decade is provided in the Del Sal review outlining which mutants GOF effects have been validated and the associated information.

Mutant p53 GOF requires the accumulation of the mutant protein and that, at least initially, it acts dominantly over the wt protein. Sabapathy discussed this important point in detail, emphasizing the timing and conditions under which dominant negative (DN) effects of mutant p53 occur, and when and how this would impact on tumorigenesis. His conclusion from the literature is that stress, whether acute (e.g., genotoxic stress) or chronic (activated oncogene), accumulates mutant p53; however, it is under the latter conditions that mutant p53 promotes tumorigenesis. Further complexity to this is the tissue specificity of the DN effect as learned from the KI heterozygote mouse models (Sabapathy). In the wake of the DN effect, there is often a loss of heterozygosity (LOH) of the wt allele. However, Walerych et al. discussed that LOH is also tissue specific, as exemplified by the work of Rotter and coworkers demonstrating that, in the embryonic stem cells of the KI mutant p53 mice, the LOH can be of either wt or mutant p53 alleles, potentially acting to control cell fate checkpoint (3). This reflects the opposing effects of wt and mutant p53 on stem cell survival and plasticity (Sabapathy).

A major requirement for GOF by mutant p53 is a constant stabilization of the mutant p53 protein, unlike the temporal accumulation of wt p53. The review by Vijayakumaran et al. summarizes the differences and similarities in the regulation of wt and mutant p53. While both wt and mutant p53 are inherently labile proteins and accumulate in response to stress, only the mutant form remains stable. Intriguingly, wt and mutant p53 share many of their regulatory mechanisms. However, the loss of the key negative autoregulatory loops due to mutation in p53 result in the sustained accumulation of mutant p53 following stress conditions or exposure to oncogenic stress in cancer cells. This, together with a loss of specificity of additional E3 ligases toward mutant p53, provides an explanation for the accumulation of mutant p53. The additional complexity of p53 regulation, both wt and mutant p53, by microRNA (miRNA) is presented Vijayakumaran et al. This reveals the ways by which p53 can be deregulated in cancer but, at the same time, may define potential new therapeutic targets.

Understanding the mechanisms by which mutant p53 gains its oncogenic functions are the subject of intensive research. While most of wt p53 activities are mediated through the transcriptional activation of target genes, the apoptotic activity of p53 also involves transcriptional independent activities. Giorgi et al. discussed the cytoplasmic apoptotic activities of wt p53, and the loss of these activities by mutations in p53. To date, there is no evidence for a GOF of mutant p53 directly regulating these activities (Giorgi et al.). On the other hand, it was reported that mutant p53 proteins can aberrantly cooperate with known transcription factors by leading to disregulated gene expression. This results in increased proliferation, invasion, genomic instability, and chemoresistance. The interaction of mutant p53 with p53 family members p63 and p73 is key to some of its GOF. Ferraiuolo et al. review the intricate relationship between mutant p53 and p63 or p73. Mutant p53 proteins can also hamper tumor suppression transcriptional programs by binding to and displacing the p53 family members p73 and p63 from their consensus of target gene promoters (4). Collectively, the intra-p53 family protein complexes with their oncogenic activity may represent druggable targets, which hold therapeutic potential.

Beyond p53 family members, Haupt et al. reviewed the major tumor suppressive pathways, which are subverted by mutant p53, including PTEN, PLK2, and PML, which control the cell cycle and the latter also the circadian clock. Intriguingly, mutant p53 deregulates cellular metabolism including glucose, lipid, and nucleotide metabolism, ensuring the sufficient supply of building blocks to support tumor growth (Haupt et al.). How are these plethora of activities achieved by mutant p53? At least two major mechanisms have been reviewed in this series. First, is by controlling gene expression through the alteration of specificity of certain transcription factors. Second, is by affecting chromatin remodeling through SWI/SNF and MLLs/MOZ (Haupt et al.). The effect of mutant p53 on MLLs/MOZ is achieved through ETS2, as reviewed in detail by Martinez. He discusses the mechanism by which mutant p53 protects ETS2 from degradation, and how this, in turn, affects the overall transcriptional effects of the ETS family and contributes to the oncogenic phenotype of mutant p53, such as increased nucleotide metabolic genes (Martinez). Bruno et al. reviewed the relationship of p53 with the cofactor Che-1/AATF. This provides an interesting example of a factor that acts as an activator and protector of both wt and mutant p53. In response to DNA damage, Che-1 induces the expression of wt and mutant p53, but activates wt p53 to induce growth arrest genes. In the case of mutant p53, it induces its expression and consequently the sequestration of p73 from apoptotic target genes, hence promotes survival (Bruno et al.).

A major consequence of mutant p53 GOFs is the acquired dependence of cancer cells on the expression of mutant p53. This dependence, which has been termed oncogenic addiction to mutant p53 has been discussed by multiple contributors to this series, highlighting its importance. Evidence for this addiction has been discussed by the Lozano, Sabapathy, and Del Sal groups. This addiction defines an Achilles Heal with important clinical implications. Parrales and Iwakuma highlighted the potential exploitation of heterozygosity, during which mutant p53 acts as a DN over wt p53, hence targeting mutant p53 eliminates its oncogenic driver and concurrently restores the tumor suppressive capacity of wt p53. Parrales and Iwakuma provided a comprehensive review of mutant p53 as a druggable target. They discuss the different classes of mutant p53 drugs, including compounds that restore wt p53 activity in cells expressing mutant p53, with the leading drug APR-246 (see below); compounds that deplete mutant p53 expression, where HSP90, in particular Ganetespib, is the most advanced drug currently in phase III clinical trial; and explore other approaches, which are currently used for other oncogenes, including knockdown and read-through of premature termination (Parrales and Iwakuma). This review was complemented by two focused reviews on mutant p53 therapeutics. The first by Bykov et al., which focused on the mechanism of action by APR-246, including the refolding of mutant p53, the impact on mutant isoforms of p53 family members p63 and p73 and the effect on the cellular redox regulators, primarily glutathione and thioredoxin, to enhance oxidative stress. The

potential of APR-246 as a single agent and in combination with DNA damaging agents is discussed, and the current clinical status of APR-246 and prospects are outlined (Bykov et al.). The second therapeutic review by Burgess et al. focused on MDM2/MDMX targeted therapies. This review provides a thorough overview of the current drugs and approaches to target p53 *via* MDM2 and MDMX pathways. They outline the clinical development of current MDM2 targeting compounds. Importantly, they discuss the major hurdle in this approach, which is severe cytopenias. Although, this approach has not been designed to target mutant p53, the relevance of mutant p53 to this therapeutic approach and the availability of appropriate biomarkers were discussed (Burgess et al.).

CONCLUDING REMARKS

Overall, this series of reviews on mutant p53 expose the pivotal role of mutant p53 as an oncogenic driver and outline the fast advancement in our understanding of its regulation and oncogenic activities. Our deeper understanding of mutant p53 also highlights clear limitations, such as the differences between mutants p53 proteins and between mouse and human mutant p53. The lack of appropriate mouse models for somatic p53 mutations, which represents the most common event in human cancer is a major hurdle to our understanding of mutant p53 to the cancer cell versus the microenvironment. The series also reflects the excitement around the clinical opportunities and current clinical development but highlights the need of potent molecules to specifically target mutant p53, given its prevalence in human cancers. It has also been increasingly clear that mutant p53 proteins are not a single entity, but they behave as a family of oncoproteins whose deciphering and therapeutic tackling might impact enormously on the success of threatening the vast majority of human cancers.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Mutant p53: Multiple Mechanisms Define Biologic Activity in Cancer

Michael Paul Kim[1,2], *Yun Zhang*[2] *and Guillermina Lozano*[2]*

[1] Department of Surgical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA, [2] Department of Genetics, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

The functional importance of *p53* as a tumor suppressor gene is evident through its pervasiveness in cancer biology. The *p53* gene is the most commonly altered gene in human cancer; however, not all genetic alterations are biologically equivalent. The majority of alterations involve *p53* missense mutations that result in the production of mutant *p53* proteins. Such mutant *p53* proteins lack normal *p53* function and may concomitantly gain novel functions, often with deleterious effects. Here, we review characterized mechanisms of mutant *p53* gain of function in various model systems. In addition, we review mutant *p53* addiction as emerging evidence suggests that tumors may depend on sustained mutant *p53* activity for continued growth. We also discuss the role of *p53* in stromal elements and their contribution to tumor initiation and progression. Lastly, current genetic mouse models of mutant *p53* in various organ systems are reviewed and their limitations discussed.

Keywords: mutant proteins, p53 mutation, gain of function, stroma, mouse models of cancer, TP53, cancer

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Edited by:
Giovanni Blandino, Regina Elena National Cancer Institute, Italy

Reviewed by:
Alessandro Rimessi, University of Ferrara, Italy
Sabrina Maria Strano, Regina Elena Cancer Institute, Italy

***Correspondence:**
Guillermina Lozano
gglozano@mdanderson.org

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MUTANT *p53*, THE ELEPHANT IN THE ROOM

Cancer is a complex disease that kills millions of people annually. Alterations in genetic and epigenetic cellular programs derail cellular controls normally responsible for maintaining homeostasis. Sequencing of human cancer genomes has identified a myriad of genomic alterations found in human cancers. Alterations in the *p53* tumor suppressor gene stand out as the most common alteration in many cancers: 96% in ovarian serous carcinoma (1), 54% in invasive breast carcinomas (2), 86% in small cell lung cancer (3), and 75% in pancreas cancer (4), to name a few. Although *p53* activity may be abrogated or lost through multiple mechanisms, the majority of these changes involve *p53* missense mutations that result in single amino acid substitutions and expression of mutant proteins. Common mutations in the *p53* gene, or "hotspots," are present; for example, approximately 86% of mutations correspond to the DNA-binding sequence of *p53* between codons 125 and 300. The predominance of mutant *p53* protein expression in human cancers over the simple loss of *p53* activity, in turn, suggests an inherent biologic advantage (5–7).

p53 BIOLOGICAL ACTIVITIES IN TUMOR SUPPRESSION

The *p53* gene encodes a transcription factor that contains a potent transcriptional activation domain, a sequence-specific DNA-binding domain, and a tetramerization domain (8). In normal cells, *p53* activity is low, but in response to DNA damage and numerous other stress signals, *p53* levels rise dramatically and result in the activation and transcription of hundreds of genes with important roles in cell cycle arrest, senescence, apoptosis, metabolism, and differentiation (9). The sum of these activities is to ensure that an abnormal cell fails to proliferate. Thus, tumors arise upon depletion

of *p53* activity through various mechanisms, including deletion or mutation of the *p53* gene itself, overproduction of the *p53* inhibitors, Mdm2 and Mdm4, and viral inactivation (10–12). Regardless of the mechanism of *p53* loss, the downstream consequences are profound and likely due to the vast, fundamental spectrum of biologic activities in which *p53* normally participates. Moreover, the loss of normal *p53* function is likely coupled with the adoption of new biologic functions exerted by mutant *p53* proteins with additional, deleterious effects.

GAIN-OF-FUNCTION ACTIVITIES OF MUTANT *p53*

Single amino acid changes in the *p53* gene may result in profound changes to its function. In human cancers, missense mutations comprise approximately 75% of all *p53* alterations (7, 13, 14). This is in contrast to many other tumor suppressor genes that undergo deletion through the course of tumor initiation or development, such as *PTEN*, *BRCA1*, and *Rb*. Five arginine residues in the *p53* gene are considered mutational "hotspots"; resultant mutant proteins fail to bind to sequence-specific DNA sites and therefore drastically alter the spectrum of transcriptional activity (15). Such signature mutations in the *p53* gene may arise through environmental exposure to ultraviolet light or chemical carcinogens such as aflatoxins, smoking, and so on (7, 16).

The fact that most *p53* alterations in tumors are missense mutations suggests that cells expressing mutant *p53* have an advantage over cells lacking *p53* (17). Numerous experiments have tested this hypothesis. For example, various tumor-derived human *p53* mutants introduced into *p53-null* H1299 lung adenocarcinoma cells conferred upon tumor cells a selective survival advantage during etoposide or cisplatin treatments (18). In addition, several *p53* mutants when overexpressed in Saos-2 cells, an immortalized tumor cell line that lacks *p53*, yielded tumors in nude mice, while the parental Saos-2 cell line did not (19). Cells expressing the most common *p53* mutants, in contrast to cells lacking *p53*, also show increased metastatic potential and invasiveness (20, 21). Mutant *p53* proteins also render some cell types more resistant to killing by therapeutic drugs such as doxorubicin, etoposide, and cisplatin (22). In Li–Fraumeni syndrome (LFS), individuals with *p53* missense mutations show a higher cancer incidence and an earlier age of tumor onset (9–15 years earlier depending on the study) than individuals with other kinds of mutations (23). These novel activities of mutant *p53* are referred to as gain of function (GOF).

The generation of *p53* knockin alleles in mice provided direct *in vivo* evidence for the GOF activities of mutant *p53*. Knockin mouse models that express mutant $p53^{R172H}$ and $p53^{R270H}$ proteins, which mimic hot spot mutations that correspond to amino acids 175 and 273 in human cancers, respectively, develop tumors that exhibit a GOF phenotype *in vivo*, with high metastatic capacity compared to tumors in mice inheriting a *p53*-null allele (24, 25). Additionally, using autochthonous mouse models of pancreatic cancer that incorporate oncogenic K-ras, Morton et al. (26) found no metastatic burden in mice that had undergone genetic deletion of a normal *p53* allele relative to a high (65%) incidence of metastasis in mice expressing a single, mutant *p53* allele (26). However, other groups that have studied identical mouse models of pancreatic cancer have found cells of pancreatic origin in the bloodstream of mice that have undergone monoallelic or biallelic deletion of *p53* in the pancreas, without the presence of mutant *p53* (27–29). These data suggest that mutant *p53* GOF activities may serve to enhance the metastatic potential and/or promote the survival and productivity of metastatic tumor cells at distant sites (26). Taken together, these studies suggest that stable mutant *p53* proteins have additional activities that fuel tumor cell proliferation and metastases that are not yet fully understood.

Interestingly, the characterization of animal models containing mutant *p53* alleles have demonstrated that tumor-specific events were required for the stabilization of mutant *p53* in addition to its simple expression. Numerous tissues derived from mouse models with germline mutant *p53* alleles failed to demonstrate detectable mutant *p53* proteins, and, in some cases, tumors failed to express detectable mutant *p53*. Investigation into this phenomenon concluded that normal tissues failed to stabilize mutant *p53* due to the presence of *Mdm2 and* $p16^{INK4a}$. Upon loss of *Mdm2 or* $p16^{INK4a}$, mutant *p53* is stabilized and mice show decreased survival and increased metastases relative to mice with intact *Mdm2 or* $p16^{INK4a}$ alleles (30). A recent analysis of pancreatic cancer specimens demonstrated a strong correlation between *p53* mutation and its stabilization through positive staining by immunohistochemistry for *p53* protein expression. Such data again indicate that in patients with pancreatic cancer, mutant *p53* proteins are expressed, stabilized, and play an important role in tumor development and progression (31). The GOF activity of mutant *p53* therefore depends largely on multiple signals for its stabilization that may vary among normal cells and even among tumor cells.

MECHANISMS OF MUTANT *p53* GOF

Several mechanisms have now been identified that contribute to mutant *p53* GOF activities. The first such mechanism discovered showed that mutant *p53* proteins abrogate the tumor-suppressive activities of the *p53* family members *p63* and *p73* (24, 25, 32–34). In addition, *TGF-β* and *EGFR*/integrin signaling pathways stabilized mutant *p53* ($p53^{R175H}$ and $p53^{R273H}$ introduced into *p53*-null H1299 cells) and inhibited the function of *p63*, properties that were essential for the invasive nature of these cells (35, 36). These studies strengthened the evidence that mutant *p53* proteins bind and disrupt *p63* activities. However, *p63* expression is limited to epithelial cells and its inhibition may therefore not explain mutant *p53* GOF in tumors of mesenchymal origin. Moreover, mutant *p53* was found to regulate gene expression independently of *p63* and *p73* in some tumors (37–40).

Using cell lines derived from these same pancreatic cancer models with *Ras* and *p53* mutations, mutant *p53* was found to drive metastasis through induction of platelet-derived growth factor receptor β (*PDGFRβ*). Mutant *p53*-dependent sequestration of *p73* from an *NF-Y* complex permits this transcriptional complex to function at the platelet-derived growth factor β promoter, resulting in expression of *PDGFRβ* and a prometastatic phenotype (41).

Chromatin ImmunoPrecipitation (ChIP)-on-chip experiments and expression arrays using SKBR3 breast cancer cells with the $p53^{R175H}$ mutation identified mutant *p53* complexes with the vitamin D receptor which augmented expression of survival genes and dampened expression of proapoptotic genes (42). Importantly, in these experiments, an intact transcriptional activation domain was required. Using expression arrays of MDA-MB-468 ($p53^{R273H}$) breast cancer cells, Freed-Pastor et al. (43) identified increased expression of genes encoding several enzymes of the mevalonate pathway. Mutant *p53* bound *SREBP* proteins and disrupted the acinar architecture of breast epithelial cells when grown as spheroids. In our studies, we compared primary osteosarcomas that had metastasized from $p53^{R172H/+}$ mice to $p53^{+/-}$ tumors that lacked metastases and identified a unique set of transcriptional changes (39). In this system, mutant *p53* bound the transcription factor *Ets2* and enhanced expression of a phospholipase, *Pla2g16*, which induced migration and invasion in culture (39). Lastly, ChIP-seq experiments using LFS fibroblasts with the $p53^{R248W}$ mutation identified numerous promoters that contain mutant *p53* (42, 44). More recently, Zhu et al. showed that *p53* mutants, not wild-type (WT) *p53*, bind to and upregulate chromatin regulatory genes, including the methyltransferase *MLL1*, *MLL2*, and acetyltransferase *MOZ*, resulting in genome-wide increases of histone methylation and acetylation. Furthermore, upregulation of *MLL1*, *MLL2*, and *MOZ* was found in human tumors with *p53* mutants, but not in WT *p53* or *p53*-null tumors (45). In summary, these data suggest that multiple pathways contribute to the GOF phenotypes of cells with mutant *p53*. The emerging themes by which mutant *p53* exhibits its GOF are (1) through formation of mutant *p53* complexes with other proteins that modify their activities (e.g., *p63* and *p73*) and (2) by interaction of mutant *p53* with other transcription factors (e.g., *SREBP* and *Ets2*) that bring a potent transcriptional activation domain to promoters not normally regulated WT *p53* (**Figure 1**). These mechanisms are not necessarily mutually exclusive in the genesis of different cancers and may be context dependent (46, 47).

DISTINCT BIOLOGICAL ACTIVITIES OF DIFFERENT *p53* MUTANTS

In addition to exhibiting GOF phenotypes, mutant *p53* proteins exhibit intrinsic differences. Some are classified as structural mutants (e.g., *p53R172H*) as the mutation alters the structure of the DNA-binding domain while others are classified as DNA-binding mutants (e.g., *p53R245W* and *p53R270H*) because they alter an arginine that directly interacts with DNA. Other mutants show partial defects. For example, the *p53R172P* mutation, albeit rare, is able to activate the cell cycle arrest but not apoptotic programs of *p53* (48). *In vivo*, differences in tumor spectrum were observed between $p53^{R172H}$ and $p53^{R270H}$ mice (24, 25). In addition, in humanized mutant *p53* knockin models, $p53^{R248Q/-}$ and $p53^{R248Q/Q}$, but not $p53^{G245S/-}$ and $p53^{G245S/S}$, mice show an acceleration of tumor development and shorter survival as compared to $p53^{-/-}$ mice (49). Lastly, different human tumor types show different spectra of *p53* mutations. For example, based on cBioPortal, mutations at the codon 248 of *p53* are most prevalently observed in human pancreatic tumors, whereas in breast tumors, codons 275 and 175 are most frequently mutated, respectively (5, 6), further suggesting that different *p53* mutations impart unique activities to drive development of different tumor types.

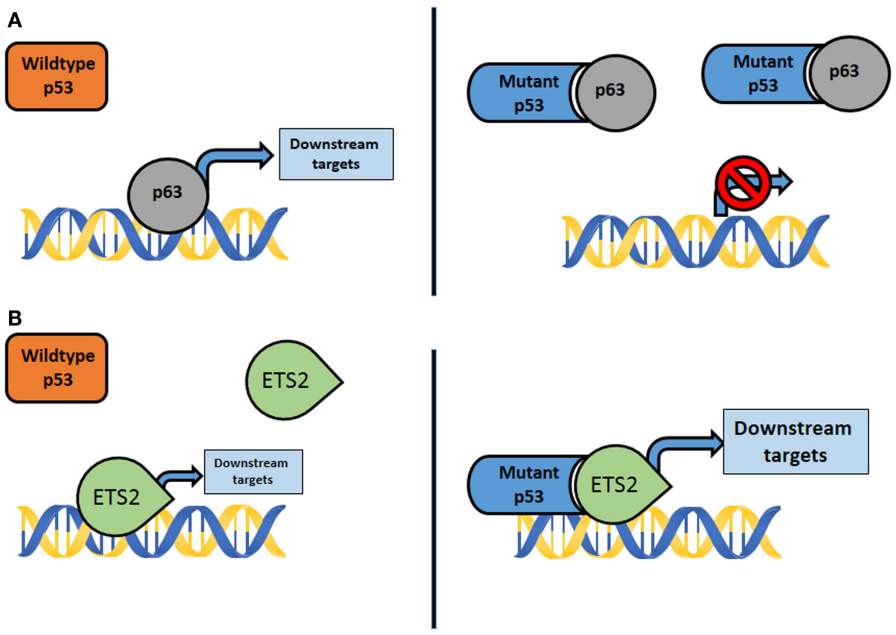

FIGURE 1 | (A) Mutant *p53* interacts with transcription factors not normally bound by wildtype *p53*, such as *p63*, *p73*, and *Smad*. The activity of downstream targets is disrupted, resulting in GOF properties. **(B)** Mutant *p53* complexes with transcription factors, such as *Ets2* and *SREBP*, not typically bound by wildtype *p53*. The results are aberrant activation of genes and downstream effectors that promote GOF properties.

THE IMPORTANCE OF STROMA IN TUMOR SUPPRESSION

The discussion has thus far focused on *p53* mutations within tumor cells and has ignored a possible role of surrounding tissue on tumor evolution. Tumors are complex tissues that consist of two components: a parenchyma and stroma. The parenchyma consists of tumor cells while the stroma consists of blood and lymphatic vessels, fibroblasts, and inflammatory and immune cells (50). The importance of stromal elements in cancer development has been supported by extensive clinical and experimental evidence (51–55). The injection of human breast tumors into nude mice and subsequent analyses of copy number variations indicated that stromal cells evolved additional changes not found in the original tumor (56). In another study of human breast cancer, gene expression differences in the stroma were a better predictor of response to chemotherapy (57). Mouse models have now clearly implicated the importance of stromal alterations in tumor development. Deletion of *PTEN* in stromal fibroblasts accelerated initiation, progression, and malignant transformation of *ErbB2/neu*-driven mammary epithelial tumors, implicating a tumor-suppressive role of *PTEN* in stroma (58). Global gene expression profiling of stroma lacking *PTEN* revealed changes in the expression of genes regulating extracellular matrix (ECM) deposition, wound healing, and chronic inflammation, which were validated by staining with various markers. Lujambio and colleagues selectively deleted *p53* in hepatic stellate cells, resulting in modifications to the tumor microenvironment (TME) and enhanced malignant transformation of epithelial cells (59). Mutations in *p53* have also been found in the stromal component of some primary breast tumors and in carcinoma-associated fibroblasts (CAFs) (51, 60–63). Additionally, MCF7 breast tumor cells formed more aggressive tumors with shorter latency after injection into $p53^{-/-}$ SCID mice as compared to injection into $p53^{+/+}$ SCID hosts (64). Hill et al. (65) further showed that prostate tumor cells can promote the selection and expansion of *p53*-deficient stromal fibroblasts through paracrine mechanisms. Highly proliferative, *p53*-deficient stromal cells were subsequently found to promote epithelial tumor growth and progression despite retention of WT *p53*. These data clearly show that changes in stroma occur and that they directly impact tumor development.

MUTANT *p53* ADDICTION

In addition to the observations that mutant *p53* proteins exhibit GOF activities, a growing body of evidence suggests that tumor cells may be addicted to mutant *p53* expression. Experiments using siRNA knockdown of mutant *p53* in cancer cell lines showed a higher apoptotic response to drug treatment in cells with knockdown of mutant *p53* (47, 66). Additional mutant *p53* depletion experiments show decreases in cell growth rate, viability, replication, and clonogenicity. Constitutive inhibition of mutant *p53* reduced tumor growth in nude mice and showed reduced stromal invasion and angiogenesis (67). In addition, Prives and colleagues showed that mutant *p53* depletion in breast cancer cells (MDA-MB-231 cells with $p53^{R280K}$ and MDA-MB-468 with $p53^{R273H}$) in 3D culture leads to phenotypic reversion to more normal, differentiated structures with hollow lumens (43). More recently, using a conditional mutant *p53* mouse model expressing a $p53^{R248Q}$ hotspot mutation, Moll and colleagues showed that mutant *p53* ablation restrained growth of allotransplanted and autochthonous tumors and extended animal survival, indicating that these tumors depend on sustained mutant *p53* expression (68). In summary, these data suggest that tumor cells with mutant *p53* may be addicted to the GOF activities of mutant *p53*. Tumor regression and dependence on mutant *p53* is likely context dependent and the extent to which elimination of mutant *p53*, genetically or through pharmacologic inhibition of downstream mediators, as a viable therapy remains to be seen (69).

MOUSE MODELS FOR SPORADIC *p53* MUTATIONS IN CANCER

Knock-in mice with germline mutations in *p53* that mimic those found in LFS have more aggressive, metastatic tumors as compared to mice lacking *p53* and provided convincing evidence for *p53* GOF activities (24, 25). Yet germline mutations in *p53* are rare in humans, and the vast majority of human cancers evolve from somatic alterations of *p53*. Consequently, current animal models at our disposal to study the role of *p53* missense mutations in the genesis of somatic tumors are inadequate. Some involve expression of mutant *p53* in breast epithelial cells using mouse mammary tumor virus (MMTV) or whey acidic protein (WAP) promoters which are hormonally regulated and therefore do not simulate expression of mutant *p53* from the endogenous locus (70, 71). Currently, conditional mutant *p53* alleles are only active following cre-mediated removal of a DNA "STOP" cassette flanked by LoxP sites (LSL = Lox–STOP–Lox) (25). The STOP sequence maintains the downstream gene in an unexpressed or null state in all animal cells until the STOP cassette is selectively removed and mutant *p53* is expressed. Importantly, tumors in this model initiate from cells heterozygous for *p53* since animal conception. Moreover, tumors under these conditions initiate and progress within a tumor microenvironment (TME) replete with *p53*-heterozygous stromal elements. CAFs, macrophages, T cells, neutrophils, endothelial cells, and so on remain heterozygous for *p53* following conception (due to the presence of the STOP cassette) with undefined effects on tumor biology. Stroma is a requisite component of tumor initiation and growth, and as mentioned earlier, prostate tumor epithelium selects for *p53*-null stromal fibroblasts in $p53^{\pm}$ mice, yielding a highly proliferative stroma that contributes to tumor progression. Given the powerful roles of *p53* in cellular plasticity and embryonic stem cell differentiation, tumors that develop from and under conditions of *p53* heterozygosity may differ in unappreciated ways from human tumors that initiate from WT *p53* cells. A model that more closely mimics the events in sporadic tumorigenesis is sorely needed.

FUTURE DIRECTIONS

Mouse models of mutant *p53* carry the potential to inform us of essential mechanisms of cancer initiation and metastasis translatable to therapeutics in humans. However, many genetic mouse models used to study mutant *p53 in vivo* incorporate germline mutant *p53* alleles that may alter normal and cancer biology in

ways that compromise its relevance to human cancer. Innovation in genetic mouse models of mutant *p53* is mandatory to more closely model human biology and to serve as translational biologic platforms to better evaluate and develop novel therapeutic agents in human cancers. Moreover, given the importance of the TME in cancer development and metastasis, mouse models that preserve the complex regulatory and tumor–stromal interactions are mandatory to the development of effective, translational biologic platforms to target the TME toward therapeutic ends.

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Mutant p53: One, No One, and One Hundred Thousand

*Dawid Walerych[1], Kamil Lisek[1,2] and Giannino Del Sal[1,2]**

[1] *Laboratorio Nazionale CIB, Area Science Park Padriciano, Trieste, Italy,* [2] *Dipartimento di Scienze della Vita, Università degli Studi di Trieste, Trieste, Italy*

Encoded by the mutated variants of the *TP53* tumor suppressor gene, mutant p53 proteins are getting an increased experimental support as active oncoproteins promoting tumor growth and metastasis. p53 missense mutant proteins are losing their wild-type tumor suppressor activity and acquire oncogenic potential, possessing diverse transforming abilities in cell and mouse models. Whether various mutant p53s differ in their oncogenic potential has been a matter of debate. Recent discoveries are starting to uncover the existence of mutant p53 downstream programs that are common to different mutant p53 variants. In this review, we discuss a number of studies on mutant p53, underlining the advantages and disadvantages of alternative experimental approaches that have been used to describe the numerous mutant p53 gain-of-function activities. Therapeutic possibilities are also discussed, taking into account targeting either individual or multiple mutant p53 proteins in human cancer.

Keywords: p53 mutation, gain-of-function, cancer, drug therapy, combination, oncogenes, tumor suppressor proteins

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***Correspondence:**
Giannino Del Sal
delsal@lncib.it

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p53 MUTANTS – ACTIVE ONCOPROTEINS

Mutations in the *TP53* gene occur in almost every type of cancer, with frequencies that vary between 10% (hematopoietic malignancies) and 96% (high grade ovarian serous carcinoma) (1). Cancer genome sequencing studies confirm that *TP53* is the most commonly mutated tumor suppressor gene in human cancers (2). The majority of studies indicate that the presence of mutated *TP53* is associated with bad prognosis in various cancer types (3). *TP53* mutations are known first and foremost to inactivate the oncosuppressive properties of the wild-type p53 protein as a transcription factor (loss-of-function – LOF). However, since p53 acts as a tetramer, expressed *TP53* mutant variants can also exert a dominant negative (DN) effect over their wild-type counterpart, and additionally they can arm cancer cells with novel oncogenic gain-of-function (GOF) activities (4–6).

In over 70% of cases, the *TP53* mutations are missense, most frequently within the region encoding the core domain of the p53 protein, which is responsible for binding DNA (7). Although the spectrum of the *TP53* missense mutations is vast – counting about 1,800 different amino-acid changes (8) – several hotspot p53 mutants, in particular, affecting residues R273, R248, R175, and G245 of the p53 protein, are present with a higher frequency both in sporadic tumors (together over 21% of total missense mutations) and in individuals with the Li–Fraumeni syndrome (LFS), a genetic disorder caused by inherited *TP53* mutations that predispose carriers to an early-onset development of various cancers (9).

The hotspot changes in p53 are traditionally classified as "conformational" or "DNA contact" mutations. This notion comes from the biophysical observation that the former group disturbs the

proper folding of the core domain of p53, thus depriving it of the ability to bind the DNA and transactivate its target genes, while the latter group is composed of mutations in residues that are responsible for directly binding DNA, with a near-native core domain structure (10, 11). In the LFS, a wild-type *TP53* allele is usually present, whereas in LFS tumors, it is often (in the 40–60% of cases) subjected to inactivation (loss of heterozygosity – LOH) – a process that is observed both in mouse LFS models (12) and in humans (13), involving various mechanisms of wild-type *TP53* inactivation (14). Interestingly, it has been recently noted that in the embryonic stem cells from LFS mice the lost allele is often the mutant one, suggesting that a bi-directional *TP53* LOH process may function as a cell-fate checkpoint and that there exists a selective pressure against the heterozygous *TP53* state (15).

p53 mutant proteins are stabilized and protected from degradation in a tumor microenvironment by various oncogenic signaling pathways (16, 17), and several studies in mutant p53 knock-in (KI) mice showed that the presence of p53 mutants promotes tumor growth with higher metastasis rate and different tissue spectrum than the absence of wild-type p53 (12, 18). These *in vivo* proofs of mutant p53 GOF came as confirmation of the initial observations in cell models that mutant p53 missense variants may actively support cell transformation (19, 20).

Even though the oncogenic activity related to GOF p53 mutants has been described many times in the last 25 years of research on p53, there are still doubts concerning its significance. Current approaches are only starting to resolve whether missense p53 mutants can be regarded as essentially one oncoprotein endowed with a conserved tumorigenic activity, or they represent a population of different oncoproteins, each exerting its unique oncogenic potential. Mutant p53 is still not used in standard clinical practice as a target of anti-cancer therapies. We discuss these issues in the following sections of this review.

ONE OR MANY – "MUTANT p53" vs. "p53 MUTANTS"

The rising importance of the GOF of p53 mutants in cancer has led to numerous studies describing their mechanisms of action and a brought forward question how much the obtained results can be generalized across different mutant p53 variants and cellular or cancer backgrounds.

A minority of these studies is based on mutant p53 KI mouse models and led to a number of discoveries in the field, including (i) the inhibitory role of mutant p53 on MRE11 protein and the induction of genomic instability (human *TP53* KI "HupKI" mouse model) (21), (ii) the transcription-based activation of PDGFRβ signaling in pancreatic cancer model (22), (ii) the transcriptional activation of oncogenic Pla2g16 phospholipase (23), and (iv) the confirmation of prior cell-based reports on a mutant p53-mediated inhibition of the p63/p73 oncosuppressive activity (12, 18). The LFS mouse-model-based studies underlined differences between GOF properties of different p53 mutants and among the consequences of *TP53* mutations in human and in mouse. Comparative studies of the R270H and R172H variants in KI mice showed different tumor spectra confirming the notion that the GOF of p53 mutants may differ (12). These spectra, however, turned out to be different also from the spectra caused by human counterparts of these mutant p53 variants – R273H and R175H – in patients with LFS (e.g., lack of mammary carcinomas in mice – frequent in humans) (9). On the other hand, the investigation in KI mice of the R246S p53 mutant, corresponding to the human R249S p53 hotspot mutant, showed no clear indication of GOF (24), whereas in human cell-based experiments, this variant was demonstrated to induce growth, chemoresistance, and a specific mutant p53 transcriptional program in several studies (25–27). Altogether these results indicate that mouse models – albeit very informative – may have their limitations and require careful confirmation of their significance in human systems.

Most of the human cell line-based studies on mutant p53 are based on initial phenotype-related experiments or large scale analysis, such as gene expression microarray or ChIP sequencing, leading to discovery of mechanisms/targets associated with a particular mutant variant in its endogenous background. In most cases, validation in other mutant p53 variants/backgrounds is also reported. Such studies have led to describing important roles of mutant p53 in direct inhibition of the p63/p73-mediated tumor suppression (28, 29), activation of the cell cycle drivers, such as Cyclins (30, 31), the vitamin D3 receptor signaling (32), steroid synthesis (mevalonate pathway) (33), the ID4-mediated angiogenesis (34), or nucleotide homeostasis (26), to name a few. A comprehensive list of these studies published since 2005 – with the indication of the initially tested mutant p53 variant(s) and p53 mutants used for validation – is shown in **Table 1**.

Mutant p53 activities have been described both in the cell's cytoplasm and in the nucleus. The reported cytoplasm-specific activities include the DAB2IP protein regulation affecting TNFα-dependent signaling (36) and the regulation of PARP localization and activity (38). Nuclear activities are related to more general influence on the chromatin function (the example of the above-mentioned MRE11 regulation) but, in most cases, are related to a specific transcriptional regulation. The available mutant p53 ChIP-sequencing data and other DNA-interaction data have not defined a mutant p53 target site analogous to that of wild-type p53, and currently the main hypothesis is that mutant p53 transactivation takes place through interaction with several transcription factors – among them NFY complex, SREBP 1 and 2, or ETS2 (**Table 1**). In most cases, mutant p53 proteins boost basic properties of these transcription factors, leading to the aberrant activation of their downstream programs and to the intersection with other key oncogenic pathways, as shown for the mutant p53-SREBP or NFY causing activation of the YAP/TAZ pathway (46, 47).

Even though experimental approaches using single initial *in vivo* and *in vitro* models led to the discovery of numerous pathways controlled by mutant p53, it is unclear whether these pathways have the same central role in diverse cellular contexts.

In an attempt to fill this gap, studies have been conducted involving the overexpression of multiple mutant p53 variants in a p53-null or wild-type background (**Table 1**). Investigation in the p53-null background of non-small lung carcinoma H1299 cells

TABLE 1 | Selected mutant p53 gain-of-function effects, mediators, and related therapeutic opportunities, published since 2005.

Mutant p53 discovered	Mutant p53 validated	Pathway(s)	Mediator(s)	Downstream proteins/genes	Leading model(s)	Mutant p53-related phenotype	Suggested treatment	Reference
R248W	R273H, R175H	DNA damage response	MRE11	AKT	HUPKI mice/MEFs	Genomic instability	–	(21)
	R175H, R280K, L194F, R273L, R249S, R248Q, C242F	DNA damage response	ETS2[a]	TDP2[a]	Li–Fraumeni-derived cell line	Chemoresistance	Etoposide	(35)
	R273L, R249S, R280K, R175H	Nucleotide homeostasis	ETS2[a]	Nucleotide metabolism genes[a]	Li–Fraumeni and breast cancer cells	Cell proliferation	–	(26)
R175H	L194F, R273H	IL-8 and GRO-α signaling	NFYA[a]	Cyclin A, B, E, CDK1, CDC25C[a]	Breast cancer cell lines	Cell proliferation	–	(30)
	R273H, R280K	Interleukin signaling,	ID4	IL-8, GRO-α	Breast cancer cell lines	Angiogenesis	–	(34)
	R273H	VDR signaling	VDR[a]	IGFBP3, CYP24A1[a]	Breast cancer cell lines	Reduced apoptosis	Vitamin D3 restriction?	(32)
	R273H, R280K	PDGF receptor β signaling	p73[a], NFY complex[a]	PDGFRβ[a]	Pancreatic cancer mouse model and cell lines	Metastasis	Imatinib	(22)
	H179R, G245S, R248Q, R249S R273H	Phospholipid metabolism	ETS2[a]	Pla2g16[a]	KI mouse model, osteosarcoma cell line	Tumor growth and metastasis	–	(23)
R280K	R273H	Cell cycle, cell movement	Pin1	Cyclin E2, BUB1, DEPDC1[a]	Breast cancer cell lines	Cell proliferation, migration	Pin1 inhibitors?	(31)
	R175H	TGFβ-induced migration/invasion	SMAD/p63[a]	SHARP-1, Cyclin G2	Breast cancer cell lines	Metastasis	–	(29)
	R175H, R273H, M237I	TNFα-driven inflammation	DAB2IP	JNK, NF-κB, and their targets	Breast cancer cell lines	Cancer-related inflammation	–	(36)
R273H	R280K	Steroid synthesis	SREBP1/2[a]	MVK, FDFT1, TM7SF2, NSDHL[a]	Breast cancer cell lines	Tumor growth	Statins	(33)
	–	HB-EGF signaling	NRD1	–	p53 null lung carcinoma	Invasion	–	(37)
	R280K, L194F	DNA replication, PARP signaling	–	PARP, MCM4, PCNA	Breast cancer cell lines	Cell proliferation	PARP inhibitors?	(38)
R175H, R273H (overexpressed)	R280K	EGFR/integrin signaling	p63	α5β1 integrin, EGFR	p53 null lung carcinoma, breast cancer cell lines	Cell motility, invasion	–	(39)
R175H, R273H, D281G (overexpressed)	–	NF-κB signaling	–	NFKB2[a]	p53 null lung carcinoma	Chemoresistance	Etoposide	(40)
R175H, R248Q, R273H (overexpressed)	R175H, R273H	Glucose metabolism, Warburg effect	RhoA/ROCK	GLUT1	p53 null lung carcinoma, MEFs, breast cancer cell lines	Tumor growth	–	(41)
R175H, R248Q, R248W, R249S, R273H, R282W (overexpressed)	R273H, R280K	Membrane and secreted signaling factors	p63[a]	DKK1, METTL7B, TFPI2[a]	p53 null lung carcinoma, breast cancer cell lines	Invasion	–	(42)
V143A, R175H, R248W, R249S, R273H, R282W (overexpressed)	R175H, R248Q, R273C	Cell cycle, apoptosis	TopBP1[a], p63/p73[a], NFY[a]	Cyclin A, B, E, CDK1, CDC25C, BAX, NOXA[a]	p53 null lung carcinoma, breast cancer cell lines	Proliferation	Calcein	(43, 44)
R175H, H179R, G245S, R248Q,R273H (overexpressed)	R175H, R273H	Ras-mediated signaling	BTG2, NF-κB[a]	CXCL1, IL1B and MMP3[a]	Human lung fibroblasts WI-38	–	–	(45)
R248Q, R249S, R273H (endogenous)	R175H, R248W	Chromatin epigenetic modification	ETS2[a]	MLL1, MLL2, MOZ[a]	Breast cancer cell lines, MEFs, Li–Fraumeni cell lines	Proliferation and tumor growth	COMPASS complex inhibitors	(27)

[a]*Transcription-related mediators and transcriptionally regulated downstream mutant p53 targets.*

led to discovering the role of mutant p53 in integrin recycling (39), in the NF-κB signaling (40), and in the Warburg effect (41) as well as a role of TopBP1 in the upstream regulation of mutant p53 (43). These studies largely confirmed that the mutant p53 GOF is exerted indirectly at the level of transcription by cooperation with transcription factors. Neilsen et al. showed that genes activated by mutant p53 largely overlap between mutant variants overexpressed in H1299 cells, but interestingly also frequently share promoter sequences with p63 and wild-type p53 (42). This indicates that the mutant p53-mediated promoter activation may be an aberrant representation of the interaction of wild-type p53 with transcription factors in normal cells. Other mutant p53 overexpression studies led to uncovering regulation of the epithelial-to-mesenchymal transition (EMT) phenotype by mutant p53 upon wild-type *TP53* silencing in MCF10A mammary epithelium cells (48) as well as the cooperation of mutant p53 with the Ras oncogenic program in WI-38 human embryonic lung fibroblasts (45). Much of this evidence, however, still awaits confirmation in experimental settings in which mutant p53 variants are endogenously expressed. During the course of transformation, cell lines carrying endogenous TP53 mutations become addicted to the mutant p53 GOF – as often their growth or migration/invasion abilities are compromised upon mutant p53 knock-down (27, 31, 36, 49). Conversely, p53-null and wild-type p53 cell lines survive and proliferate without mutant p53, suggesting that very likely the GOF program observed under such conditions only partially resembles the cancer-related one. Therefore, the lack of the cellular context in which p53 mutants are naturally embedded and background-associated effects represent relevant weaknesses of the studies in a p53-null or p53 wild-type background.

A solution to these limitations may be represented by studies that include an initial analysis using different p53 mutants in their endogenous backgrounds. Analyzing downstream programs – both at the phenotypic and the molecular level – may help to understand to what extent p53 mutants possess a "core" oncogenic program, and whether some mutants display specific features. A recent study by Zhu et al. focuses on the common DNA interaction pattern of three distinct p53 mutants, in their endogenous context of breast cancer cell lines, using as term of comparison the pattern obtained from two cell lines bearing wild-type p53 (27). As a highlight of this multi-mutant p53–DNA interaction pattern, the group identified the chromatin regulatory genes that are activated by the transcription factor ETS2, a previously known mutant p53 interactor (23, 26, 35). The relevance of a mutant p53/ETS2 cooperation has been confirmed as a general feature in several mutant p53 expressing cell lines and thanks to the transcriptional program perturbed, as a critical modulator of the chromatin modification (27).

Even with these many studies published this is apparently only the beginning of a deeper understanding of both specificity and general picture of mutant p53 GOF in cancer. Multiple cellular/cancer models have to be studied simultaneously in unbiased, large-scale manner, by comparing more mutant p53 variants, including non-hotspot mutations. Another important issue is how these discoveries could be transferred into clinical applications.

TARGETING MUTANT p53 IN CANCER

The issue regarding how widely the GOF effects are shared between multiple mutant p53 variants extends to the experimental targeted therapies based on the presence of mutant p53. Since *TP53* is one of the most frequently mutated genes in cancer, reactivation of the wild-type p53 oncosuppressive properties and eliminating the mutant p53 GOF are potentially instrumental in personalized treatment of hundreds of thousands cancer patients worldwide. In this context, the possibility to distinguish mutant p53-specific processes from those shared by at least hotspot mutant p53 variants seems of relevance in order to develop and test drugs targeting properties and/or downstream pathways that are common to as many mutant p53 variants as possible.

Research on widely acting molecules targeting mutant p53 began over two decades ago. Some of the first approaches included inhibitors of Hsp90, a molecular chaperone that participates in a multiprotein complex stabilizing GOF p53 mutants with distorted DNA-binding domain structure (50). Hsp90 inhibitor geldanamycin was shown to lower levels and nuclear translocation of mutant p53 (51, 52). The interest toward Hsp90 inhibitors remains high, as the recent study by Alexandrova et al. describes significantly increased survival of mutant p53 KI mice treated with the geldanamycin derivative 17-DMAG or with a new generation Hsp90 inhibitor – ganetespib (53). Other drugs – such as the histone deacetylase inhibitor SAHA (Vorinostat) (54) and sodium butyrate (NaB) (55) – have been also shown to downregulate the levels of mutant p53 variants.

Different suggested strategies involve blocking the mutant p53 activation by targeting proteins, such as Pin1 (31) or TopBP1 (43). Inhibitor of TopBP1 – Calcein (44) – and experimental inhibitors of Pin1 (56) are examples of molecules targeting specific upstream activators of mutant p53. Among compounds that have been shown to efficiently directly target mutant p53 are small peptides (57–59). None of them, however, is so advanced in experimentation as small molecules that directly modify mutant p53 promoting its transition into a wild-type like form, capable of activating the tumor suppressive wild-type p53 transcriptional targets. The first described micromolecule targeting mutant p53 was CP-31398 (60) that, despite turning out not to directly interact with mutant p53 but rather with its target DNA sequences (58), is still considered as a promising drug candidate (61, 62). Most studies were, however, performed on the PRIMA-1 molecule (63) and later on its more potent and less toxic derivative PRIMA-1MET/APR-246 (64). Experiments showed that this molecule is able to directly bind and modify thiol residues in mutant p53 transforming it into a wild-type-like protein (65), thus becoming able to activate wild-type p53 targets, such as *GADD45B*, *NOXA*, or *CDKN1A* (p21), and induce *in vitro* and *in vivo* cell cycle arrest or apoptosis (66, 67).

In the case of drugs targeting mutant p53, most studied molecules, as those mentioned above, target several mutant p53 missense variants, while drug candidates focusing on particular mutants are rare. NSC319726 is one such these compounds. Identified by screening studies, NSC319726 possesses specific activity toward the R175H mutant p53 and induces apoptosis

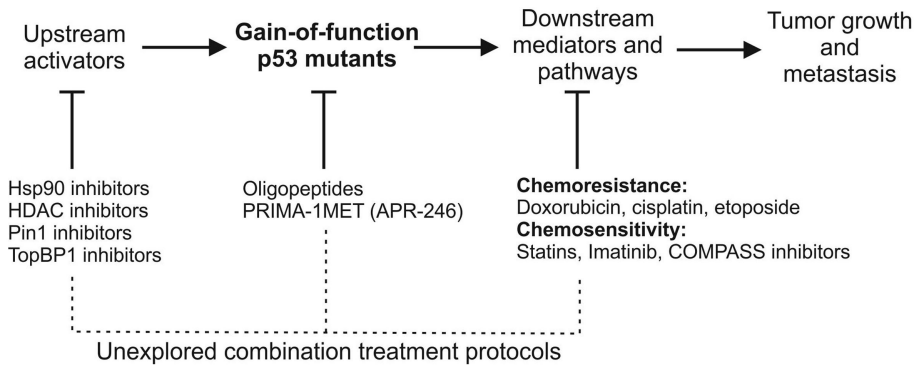

FIGURE 1 | A schematic view of gain-of-function mutant p53 activation, mutant p53 downstream effectors/pathways, and therapeutic opportunities of targeting each of the processes. Below the largely unexplored possibilities of mutant p53-related combinational anti-cancer therapies are suggested.

in human cells (68). Two other studies led to discovering PhiKan083 (69) and PK7088 (70) as molecules that specifically target and reactivate mutant p53 hotspot variant Y220C, which is found at a relatively high frequency in breast cancer (5). The low number of such studies and the fact that other mutant p53 reactivating compounds target various mutant p53 variants may suggest that the classic distinction of contact and structural p53 mutants may not be decisive and these mutant types may, in fact, represent structural extremes of a spectrum of distortions in the DNA-binding domain, leading to similar GOF effects.

Another important strategy to mutant p53 targeting is based on the treatment with drugs that downregulate oncogenic pathways activated by the means of mutant p53 GOF (listed in **Table 1**). This activation in general leads to two types of therapeutically relevant outcome – chemoresistance and chemosensitization (**Figure 1**). In the first case, the sensitivity to either specific or broad activity anti-cancer compounds, including doxorubicin, cisplatin, or etoposide, is dampened in the presence of mutant p53 (35, 49). In the latter case, a number of pathway targeting drugs – such as statins that inhibit the mevalonate pathway (33), imatinib inhibiting PDGFRβ (22), or COMAPSS complex inhibitors (27) – can cause increased cell death in mutant p53 vs. wild-type p53-bearing cancer cells. The performance of these drugs is often promising, but their drawback is the limited number of mutant p53/cell backgrounds tested.

A big issue of the mutant p53-oriented therapies is their slow progress toward the clinics, most of them being still at an early stage of development (71). The only drug directly targeting mutant p53 that has reached the clinical stage is PRIMA-1MET/APR-246. This compound successfully went through phase I/II clinical trial in hematological malignancies and prostate cancer that included mutant p53 patients (72). An approach targeting triple negative breast cancer (TNBC) cells with p53 deficiency or mutant status using Chk1 inhibitors showed promising results in *in vitro* and mouse tests (73, 74), while it failed to show significant improvement in human patients (75). At the same time, many of the drugs that could be beneficial for mutant p53 patients – Hsp90 inhibitors, HDAC inhibitors, or statins – are undergoing clinical trials in cancer in which the mutant p53 status is not considered or even known (76–78).

The combination of drugs directly targeting mutant p53 with drugs inhibiting mutant p53-related pathways is surprisingly avoided (**Figure 1**), although it might favor the decrease of compensatory responses and dosage toxicity, and thus an increase in the therapeutic efficacy. This notion is supported by a number of experiments showing that the combination of PRIMA-1 and PRIMA1-MET/APR-246 with cisplatin (CDDP) results in synergistic effects in cancer cells and xenografts (79–81). Taking into account that mutant p53 is known to increase chemoresistance to cisplatin (49), it is not surprising that targeting the cause of this chemoresistance opens the window to more effective treatments. This combinational approach may suggest that other compounds are worth being tested together with mutant p53 targeting drugs, such as PRIMA-1MET/APR-246 (**Figure 1**).

Even though *TP53* is one of the most frequently mutated genes in human cancer and mutant p53 emerges as a major oncoprotein controlling an exceptionally vast network of tumor-promoting activities, it still possesses underused potential as a drug target and much effort is needed to bring it to a prominent position on the map of personalized therapeutic solutions for cancer patients.

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REVIEW
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The Contrived Mutant p53 Oncogene – Beyond Loss of Functions

Kanaga Sabapathy[1,2,3,4]*

[1]Division of Cellular and Molecular Research, National Cancer Centre Singapore, Humphrey Oei Institute of Cancer Research, Singapore, [2]Cancer and Stem Cell Biology Program, Duke-NUS Graduate Medical School, Singapore, [3]Institute of Molecular and Cellular Biology, Singapore, [4]Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

Mutations in *p53* are almost synonymous with cancer – be it susceptibility to the disease or response to treatment – and therefore, are a critical determinant of overall survival. As most of these mutations occur in the DNA-binding domain of p53, many of the clinical correlations with mutant p53 have been initially relegated to the loss of its transcription-dependent activities as a tumor suppressor. However, significant efforts over the last two decades have led to the vast knowledge on the potential functions of the mutated p53 protein, which have been attributed to the physical presence of the mutant protein rather than the loss of its wild-type (WT) functions. Beyond the inhibitory effects of mutant p53 on the remaining WT protein that leads to the dominant-negative effect in the heterozygous state, mutant p53's presence has also been significantly attributed to novel gain-of-functions that lead to addiction of cancer cells to its presence for survival, as well as for their ability to invade and metastasize, elevating it to a contrived oncogene that drives the cancer cells forward. This review will summarize the functional consequences of the presence of mutant p53 protein on cellular and organismal physiology.

Keywords: dominant-negative effect, gain-of-functions, mutant p53-addiction, oncogene, p53, tumor-suppressor

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***Correspondence:**
Kanaga Sabapathy
cmrksb@nccs.com.sg

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MUTANT p53

p53, one of the earliest identified tumor suppressors, has been overwhelmingly confirmed to be the most mutated gene across all cancer types through the recent avalanche of cancer genome sequencing efforts (1). Mutations in *p53* occur in about 90% of the cases in its central DNA-binding domain (DBD), thereby leading to loss of its transactivation properties that are often associated with its tumor-suppressor functions [reviewed in Ref. (2)]. During the course of the evolution of the transformed cell, mutant p53 derived from the mutated allele co-exists with the wild-type (WT) p53 from the other allele for varying time periods, till the WT allele is generally lost through loss-of-heterozygozity (LOH), resulting in the sole existence of only the mutant p53 (**Figure 1**). Like most of the tumor suppressors that have a direct impact on tumor growth upon their loss of function – thus qualifying them as tumor suppressors – mutations in *p53* were also thought to lead to loss of most of its tumor-suppressor functions that regulate almost all aspects of cellular physiology. Interestingly, during the co-existence phase of both the WT and mutant p53 proteins, haploinsufficiency leads to propensity for increased tumor development, as has been demonstrated in Li–Fraumeni patients

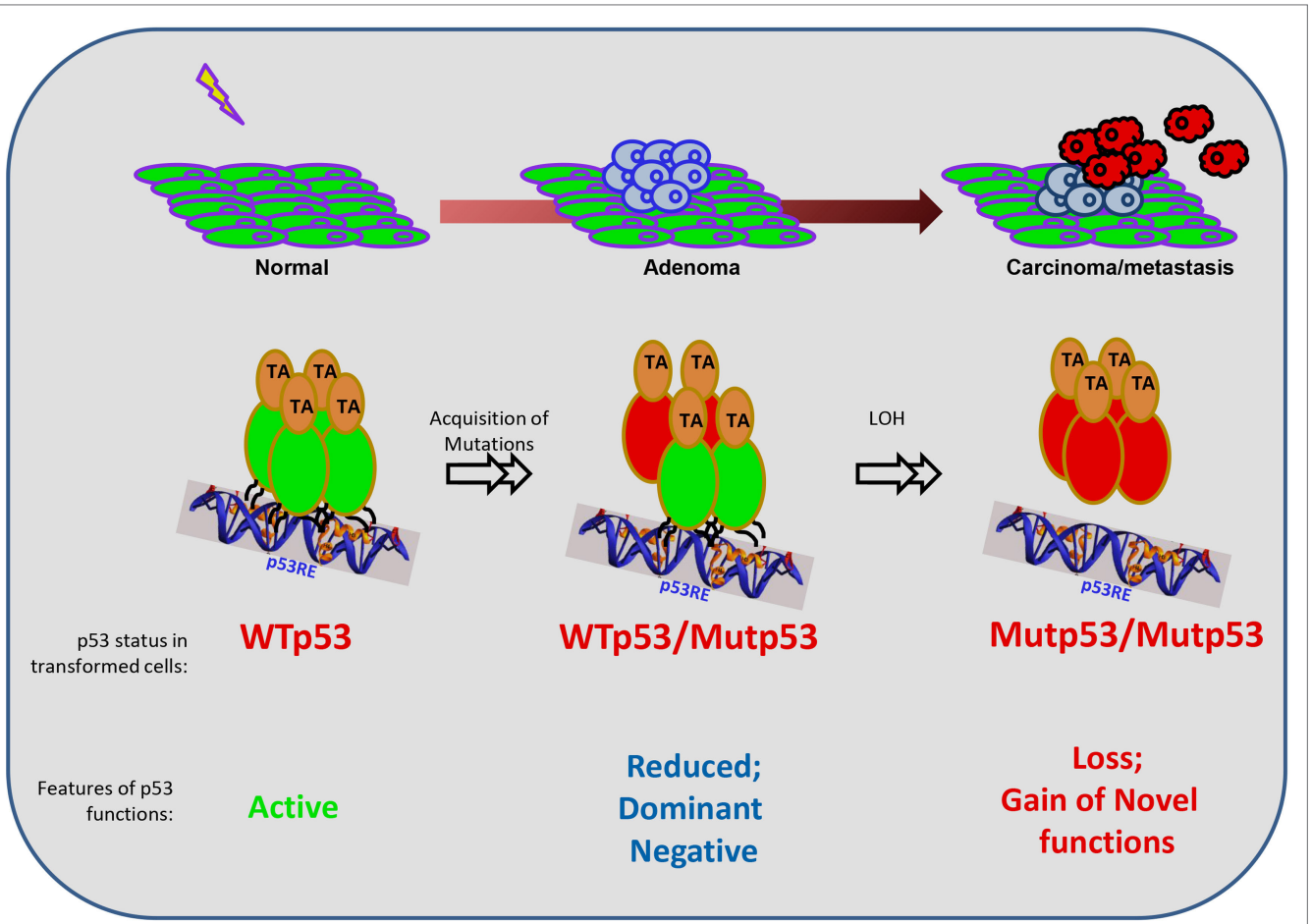

FIGURE 1 | Mutant p53 functions during the evolution of a cancer cell. The schematic represents the general evolution of a normal cell into a transformed cell (carcinoma), and the contexts in which mutant (Mut) p53 exerts its functions. *p53* mutations are not present in the normal case and are induced upon genotoxic exposures in one allele. Hence, in the intermediate stage, the mutant p53 co-exists with the wild-type (WT) p53, until the loss of the wild-type allele by loss-of-heterozygozity (LOH). Functionally, when p53 is unmutated, it can be activated and works as a tetramer. However, when one allele is mutated, there is reduced overall function resulting in haploinsufficieny, and also the dominant-negative effect of the mutant protein on the wild-type protein due to the formation of heterodimers (please see text for details). At the later stages when only the mutant p53 remains, it is unable to bind to canonical target sequences to turn on its targets, leading to loss of wild-type functions. In addition, mutant p53 acquires novel gain-of-functions to drive the growth, survival, and invasion of tumor cells.

as well as in a large number of model organisms expressing the mutant *p53* allele (3, 4). However, in contrast to most tumor suppressors, mutant p53 is unique in the sense that it has not only lost its tumor-suppressor functions, but evidence accumulating over the last two decades has assigned a wide variety of advantages to the cancer cell of having the mutant p53 protein. In short, the advantages of having a mutant p53 to the growth and survival of the tumor cells can be classified as a mirror image of the tumor-suppressor functions of WT p53 (**Figure 2**). Of interest is the fact that no other tumor suppressor has acquired such a wide array of novel functions as p53 when mutated, thereby elevating p53 to the status of a "contrived" oncogene, entirely on the basis that its functions as a tumor suppressor are turned on its head when the gene gets mutated. In this review, I will discuss the novel and acquired functions of mutant p53, specifically focusing on the DBD mutations.

DOMINANT-NEGATIVE FUNCTIONS OF MUTANT p53 – AT THE EARLY STAGES

Generally, in the early phases of cancer development, mutated *p53* co-exists with the WT allele until the latter is lost due to LOH. In this co-existence phase, haploinsufficiency is a generally observed phenomenon associated with tumor development (5). However, effects of the mutant p53 over the WT's regular functions – in a dominant-negative (DN) manner – have also been noted. Several early studies indirectly illustrated this DN effect, especially through the overexpression of the mutant p53 in WT p53 expressing cells, or vice versa. However, direct evidence for the DN effect of the mutant p53 protein on the WT p53 was shown in co-overexpression studies, demonstrating the quenching of WT p53's ability to affect cellular transformation and transactivation of target genes, especially in transformed cell lines (6,

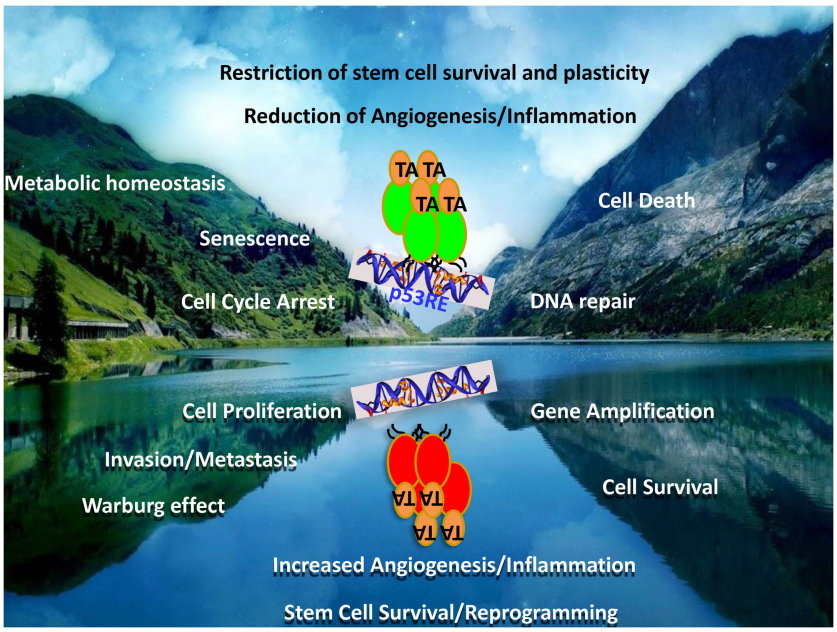

FIGURE 2 | Mutant p53 – the contrived oncogene. The figure represents a mirror image of the functions of the wild-type and mutant p53 proteins. While wild-type p53 is a tumor suppressor, the mutant form represents not only a loss of these functions but also the acquisition of directly opposite functions. Many of the tumor-suppressor functions and the counteracting oncogenic functions by mutant p53 are represented as mirror-image pairs: cell death/cell survival; cell cycle arrest/cell proliferation; DNA-repair/genomic instability; senescence/invasion and metastasis; metabolic homeostasis/Warburg effect; restriction of angiogenesis and inflammation/increased angiogenesis and inflammation; restriction of stem cell plasticity and survival/increased reprograming and expansion, to highlight a few.

7). Co-expression of WT p53 with mutant p53 was also shown to affect the conformation of the former into a mutant conformation due to co-translation with the mutant form (8). Moreover, WT p53 was suggested to be inactivated through oligomerization with mutant p53 (9), and by inhibition of WT p53's ability to bind to target gene promoters in a specific manner (6, 10). Concomitant to the effects on target gene activation, DN effects were noted on WT p53's ability to induce cell death (11) and ras-induced senescence (12). Consequently, large efforts to analyze the DN effects of tumor-derived p53 mutants on the activation of several target genes have been undertaken in a systematic way using the yeast model, which was able to classify mutations as either dominant or recessive (13), and has also led to the cataloging of p53 mutations based on the ability to regulate a large number of p53-target genes (14).

Although the DN phenomenon has been well demonstrated in a large series of studies, the question that remained was its relevance when mutant p53 is expressed from its endogenous locus. This was resolved with the generation of the p53 mutant knock-in mouse models, whereby several groups confirmed the DN effects using $p53^{mutant/+}$ mice in various primary cell types, including thymocytes, splenocytes, and embryonic stem cells, by comparing it with the $p53^{-/+}$ cohorts (15–18). Interestingly, while the DN effects were seen in some tissues, both on target gene activation and on cell survival, this was not the case in other cell types, as in primary fibroblasts in their growth, suggesting that the DN effect might be cell type, and possible stimulus dependent (18).

An interesting observation that emerges from all these studies with primary and transformed cells is that the DN effects on target gene activation and cell death are generally seen when cells are exposed to stress stimuli, including exposure to DNA-damaging agents, when p53 is activated and stabilized (15, 16, 18). By contrast, DN effect is not normally observed at baseline conditions, as there were no growth advantages to primary cells from $p53^{mutant/+}$ mice, or with respect to spontaneous tumor development, both of which mirrors the $p53^{-/+}$ cells and mice (18, 19). Furthermore, although the DN effect can lead to almost complete ablation of target gene activation when observed, all $p53^{mutant/+}$ mice generated so far have not been able to rescue the embryonic lethality due to the absence of Mdm2 (15, 18). Thus, these data collectively surmise that the DN effect of mutant p53 is exhibited when the levels of the mutant p53 is elevated in acute stress conditions, and thus, may have a significant consequence when patients are treated with chemotherapy and radiotherapy. However, a noteworthy point is that DN effects are not seen with respect to susceptibility to tumor formation, even in the case when $p53^{mutant/+}$ mice have been irradiated, supporting the notion that DN effects observed upon acute activation of p53 that affects short-term apoptotic response do not have a contributory role to the long-term tumorigenic effects (18). Thus, acute p53 activation and DN effects of mutant p53 can be decoupled from the long-term effects of p53 in regulating tumor susceptibility.

In this respect, early reports have suggested that at least three molecules of mutant p53 are required to impose a DN effect on one molecule of WT p53 (7, 20). Interestingly, although mutant

p53 protein has been shown to have a much longer-half life than WT p53, especially in tumor cell lines and in transfection studies, primary cells and tissues from $p53^{mutant/+}$ mice do not exhibit elevated steady-state levels of the mutant p53 protein (18, 19). However, mutant p53 appears to be more abundant in tumor tissues from these mice, further alluding to the requirement of stress and/or oncogenic signals for mutant p53 stabilization (18, 19). Given that stress signals are also able to further stabilize mutant p53, it is not unexpected that the ratio of mutant p53 to WT is significantly high, thereby leading to the observed DN effects. In supporting this theory, reduction of the endogenous mutant p53 levels – using an hypomorphic mutant p53 knock-in mouse model – was indeed shown to alleviate the DN effects, both on target gene activation and cell death upon irradiation (18). Similarly, reduction of the WT p53 levels in a $p53^{mutant/+}$ mice strain also led to exhibition of the DN effect in tumors (21). Thus, these observations consolidate the case for the requirement of a significant increase in mutant p53 levels – as seen in tumor cells, or in primary cells upon exposure to stress stimuli – for the manifestation of the DN effects, which could be ameliorated by reducing the mutant protein levels. This implies that in a clinical setting, strategies that would reduce mutant p53 protein levels without an effect on WT p53 during therapy would lead to better efficacy of treatment and would be a future prospect that should be followed up.

Mechanistically, there are a few modes of operation of the DN effect. The mutant p53, which itself is unable to specifically bind to the p53-response elements, binds to the WT p53, thereby quenching it away from target gene promoters (6, 10). Alternatively, mutant p53 quenches away co-factors that are required for transactivation by the WT p53 bound to the promoter, thereby reducing the potency of the WT protein (22). In addition, mutant p53 has been suggested to form aggregates, akin to those seen in protein misfolding diseases. Herein, it has been suggested that the WT p53 protein is engulfed into mutant p53 fibrillar and granular aggregates, whereby the misfolded mutant protein sequesters the WT form, thus leading to inactivation of the WT function (23). Whatever the mechanism may be, the underlying concept is that the ability of mutant p53 to bind to wild-p53 when in excess is causal to the DN effect, which could thus offer an avenue for exploitation for therapeutic benefit. While there are currently no known ways of overcoming the DN effect, potential strategies that lead to the degradation of mutant p53 specifically without affecting the WT protein will be the way forward in ameliorating the DN effect.

AFTER LOH – THE MUTANT p53-ADDICITON PHENOMENON

While the phenomenon of addiction to oncogenes has been well established (24), mutated tumor suppressors have never been earlier reported to provide a survival advantage to tumor cells due to any novel acquired functions. However, two reports in the mid-2000s showed for the first time that silencing the expression of endogenous mutant p53 can lead to increased apoptosis (25), and reduced tumor growth *in vivo* (26), formally demonstrating the phenomenon of addiction of tumor cells to mutant p53's presence. In addition, indirect evidence for the requirement for mutant p53 for survival of cells in a phospholipase D-dependent manner was also noted (27). This phenomenon is now well established in a large number of subsequent studies. However, the causal mechanisms are still relatively elusive. In earlier studies, a role for transactivation by mutant p53 of cell growth regulation genes was suggested, as the transactivation deficient DBD p53 mutants were unable to provide a growth advantage (28). In the other studies, mutant p53-mediated suppression of canonical p53-target genes was suggested to be the mechanism, which was ameliorated upon the silencing of mutant p53 expression, leading to cell death. In this latter case, hypomethylation appeared to be involved, as trichostatin A – a HDAC inhibitor – was found to relief the mutant p53-dependent suppression (25). Other recent studies have confirmed this possibility, using other HDAC inhibitors, such as SAHA (29) and sodium butyrate (30).

Recent *in vivo* work in mice has also confirmed that destabilization of mutant p53 expression leads to apoptosis and reduction of tumor growth (31). In this case, mutant p53 was destabilized through the inhibition of the HSP90/HDAC6 chaperone machinery that is often upregulated in cancers, collectively highlighting the mutant p53-addiction phenomenon, and that degradation of mutant p53 can indeed enhance tumor cell death and improve therapy. While addiction to mutant p53 appears to be critical for the survival of the cancer cell, the exact point at which they get addicted to mutant p53 is not understood. While the transformed cells could be expected to become addicted to the presence of mutant p53 at the point in time of loss of the remaining WT p53 allele, it is likely that further events are required for this phenomenon to occur. Moreover, whether addiction to mutant p53 is a universal phenomenon in all cell types also requires further systematic analyses.

ROLE REVERSAL FROM TUMOR SUPPRESSOR TO ONCOGENE: GAIN OF NOVEL FUNCTIONS

Similar to mutant p53 addiction, the direct advantages conferred by the presence of the mutant p53 protein have been understood primarily through cell culture studies where isogenic cell lines without p53 expression have been used to analyze the effects of the overexpressed mutant p53. These have resulted in the uncovering of a plethora of gain-of-function (GOF) effects, almost all of which provide survival/growth advantage to the cell. First direct evidence came from the overexpression of several human (e.g., R175H, R248W, R273H, and R281G) and mouse mutants in p53 null cell lines that lead to increased cellular growth in soft agar and increased tumorigenicity in immunocompromised mice (32). While this was the first case of evaluating the effects of the mutant version of the natural tumor suppressor, earlier studies using a mutant p53 – at that time thought to be the natural existing form prior to the knowledge that p53 is actually a tumor suppressor – also showed growth advantage due to its overexpression (33). In addition, there are multiple other parameters associated with genomic instability that were noted due to the

overexpression of mutant p53, including gene and centrosome amplification and disruption of spindle checkpoint (34–37). Consistently, overexpression of mutant p53 also led to resistance to death induced by a variety of chemotherapeutic drugs and DNA-damaging agents (38–41), as well as by anoikis (42). More recently, expression of mutant p53 was also shown to enhance the Warburg effect on cancer cells, promoting GLUT1 translocation to the plasma membrane, and thus enhancing glucose uptake (43).

Not unexpectedly, many studies have also evaluated if the expression of mutant p53 would enhance cellular invasion and migration and found that to be the case in a variety of 2D and 3D cellular systems (18, 44–46). Furthermore, a role for mutant p53 in promoting cellular reprograming was also demonstrated (47), suggesting that mutant p53's presence would lead to the survival and replenishment of the potential cancer stem cells, leading to their ability to colonize the adjacent territory.

At the organismal level where mutant p53 is expressed from its own locus – reflecting the status in human cancer conditions – GOF properties were also noted with the generation of the p53 mutant knock-in mice. The initial data demonstrated that the $p53^{R172H}$ mice (equivalent to human R175H) were more tumor prone with more carcinomas – reflecting a change in tumor spectrum. They also had increased metastasis compared to the p53 null mice, in the absence of Mdm2, which leads to increased mutant p53 levels (15, 16). Further studies have cemented these findings, where the presence of the R172H mutant p53 protein conferred significant growth and metastatic propensity to tumors compared to the loss of p53, in several oncogene-induced models, including Ras and APC, in a variety of tumor types such as lung and pancreatic ductal adenocarcinomas (46, 48, 49). Similar observations were also noted with other mutations, such as the R270H and R273H in breast and lung cancer models (50, 51). These data mooted the idea that all mutant p53 would have GOF properties, and that this may depend on the stabilization of the mutant p53 in the cancer cell context, as normal untransformed tissues from the mutant p53 bearing mice did not have significant growth advantage and did not display increased steady-state levels of the mutant protein (19). However, this theory came under challenge through the analyses of another hot-spot p53 mutant knock-in mouse strain (the R246S, equivalent to human R249S). In this case, the R246S mutant mice did not display any tumor latency difference or metastatic advantage even in the absence of Mdm2 (18). Similar results were also seen with other hot-spot mutant knock-in mice strains, in which the R248Q had a strong GOF phenotype in contrast to the G245S mutant (52), collectively alluding the fact that GOF may not be a universal phenomenon, and that elevation of mutant p53 levels may not be sufficient for their exhibition.

Nonetheless, as observed in the cell culture-based studies, there was a propensity to have more hematopoitic or mesenchymal stem cells in the R248Q mutant p53 knock-in mice, indicative of an effect of mutant p53 on the plasticity of the stem cell population (52). Besides the effects on tumor metastasis and aggressiveness, the effects of mutant p53 on several other aspects of physiology have been noted using the knock-in mice strains. For instance, there was increased inflammation and tissue damage, primarily due to upregulation of inflammatory cytokines that were induced by mutant p53-mediated prolonged activation of NFKB signaling (53). Angiogenesis was also shown to be enhanced due to mutant p53 expression, through the activation of ID4 expression in cell culture studies (54). These studies collectively indicate that expression of mutant p53 in tumor cells, as seen in the case with human cancers, as well as in normal tissues as analyzed from animal studies, has far-reaching consequences on organimsal physiology.

While enormous amounts of data from cellular systems and animal models highlight the existence of GOFs of mutant p53, albeit to varying degrees perhaps depending on several contextual factors, the main question is the relevance of this phenomenon in humans. A noteworthy point is that humans generally do not carry a mutant *p53* allele in non-transformed tissues, except in the case of the Li–Fraumeni patients. Thus, the GOF properties would be of relevance in the large majority of tumors that eventually retain only the mutant allele. On the other hand, the Li–Fraumeni patients would be expected to have one allele that is WT in all untransformed tissues, except in cases where LOH would result in or occur with other transforming events that lead to tumorigenesis. In this context, transcriptomic analyses of $p53^{-/-}$ vs. $p53^{mutant/mutant}$ primary tissues (e.g., thymus) from the knock-in mice remarkably did not reveal any significant changes (18), highlighting that GOF is generally not observed in untransformed cellular contexts. Thus, even in the Li–Fraumeni group, the GOFs and addiction to mutant p53 would be a phenomenon that would be of relevance primarily in the cancer cell context. This has been exemplified in a large number of studies that have evaluated the role of mutant p53 in response to chemotherapy, which have generally shown poor prognosis associated with the presence of mutant p53 (55). This, however, could either be due to the DN effect or the GOF functions, as many of these reports have not teased out the status of the other allele in the patient samples. Thus, the presence of mutant p53 in human tumors does indeed exert a negative effect in response to therapy, warranting in-depth investigations into the mechanisms of actions of mutant p53.

MECHANISMS AND MEDIATORS OF MUTANT p53 GAIN-OF-FUNCTIONS

Since the demonstration of GOF of mutant p53, extensive efforts have gone on to examine the mechanistic basis of this phenomenon [reviewed in Ref. (56)]. At least four inter-related categories of actions have emerged: novel target gene activation through direct DNA binding; enhancement of target gene activation due to co-factor binding to mutant p53; co-operation with other transcription factors to activate other transcriptomes; and binding to factors that indirectly result in activation of other pathways.

In the first instance, while DBD-mutant p53 has lost its abilities to bind to canonical p53-responsive elements on target gene promoters, several studies have alluded to the ability of mutant p53 to bind to novel target gene promoters to activate them. It has been proposed that mutant p53 interacts with matrix-attachment regions (MARs), thereby recognizing structures rather than

sequences (57). This results in the activation or repression of mutant p53-target genes, the list of which has been steadily growing. In brief, the targets can be categorized into either genes that are upregulated or down-regulated by the presence of mutant p53, including both coding and non-coding genes involved in promoting proliferation, inhibiting cell death, promoting migration, and inflammation. In the former category exists a large array of genes, including growth factors/receptors, such as bFgF, Egfr, Igf1, Igf1r, IL6, and TNFα; cell death/survival genes, such as bcl-xl, procaspase 3; oncogenes and transcription factors, such as c-fos; c-Myc, Egr1, Nfkb, Ras, and Egr1; metastasis regulators, such as twist-1; microRNAs, such as mir 155 and mir128-2; and many others [reviewed in Ref. (2)]. The second category of suppressed genes includes Fas, Mst-1/msp, mir130b, mir27a, mir 223, and also a large array of the canonical p53-target genes that appear to be suppressed by the presence of mutant p53 (25).

Next, regulation of target genes by mutant p53 can be facilitated by co-operative binding and post-translational modifications of the mutant p53 protein. For instance, mutant p53's activity has been shown to be enhanced by binding with PML and Pin1 (58, 59). In addition, mutant p53 has also been shown to co-operate with other transcription factors, such as NF-Y, VDR, Sp1, and SREBP, to enhance the activation of their target genes (60–63). Conversely, mutant p53 has also been shown to bind to p63 and p73 to inhibit the activation of the latter groups' targets (64). Finally, an indirect role for mutant p53 in activation of various pathways has emerged. For example, mutant p53 has been shown to bind to p63, thereby negating the latter's inhibitory effects on the α5β1/RCP complex, which leads to enhanced cellular motility (46). Similarly, mutant p53 has been shown to bind to proteins in the DNA-repair pathway, such as MRE11, thereby affecting their functions, which leads to increased genomic instability (65).

The diversity in mechanisms and mediators of mutant p53's GOF function allude to the fact that these may be dependent and vary according to the cell type, the mutation type or the stimuli. Furthermore, GOFs would also likely be temporally dictated during the evolution of the transformed cell. Hence, efforts aimed at targeting mutant p53 would have to take into account these factors that have to be elucidated and characterized.

FUTURE OF MUTANT p53

One can envisage that all the years of work on mutant p53 and its functions will now put the research community in good stead in trying to target its functions for clinical benefit, and the current status of these efforts is reviewed in the adjoining chapters. Nonetheless, important considerations have yet to be worked out. These include several questions, such as how can the DN effect be mitigated when treating patients whose tumors retain the WT allele; what is the effect of the DN phenomenon in the daily lives of LF patients, especially when they are exposed to a variety of signals that may have an acute effect on p53 activation; precisely when and where is GOF manifested and addiction to mutant p53 occur; and what is the trigger point for GOF of mutant p53. Thus, while general targeting strategies are being worked out, more work is required to realize the dream of targeting all types of mutant p53 that are different, and thus, likely require specific molecules/dugs to counter them.

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KS planned and wrote the manuscript.

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Regulation of Mutant p53 Protein Expression

*Reshma Vijayakumaran[1], Kah Hin Tan[1], Panimaya Jeffreena Miranda[1], Sue Haupt[1] and Ygal Haupt[1,2,3]**

[1] Tumour Suppression Laboratory, Peter MacCallum Cancer Centre, Melbourne, VIC, Australia, [2] Sir Peter MacCallum Department of Oncology and Department of Pathology, The University of Melbourne, Parkville, VIC, Australia, [3] Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC, Australia

For several decades, p53 has been detected in cancer biopsies by virtue of its high protein expression level which is considered indicative of mutation. Surprisingly, however, mouse genetic studies revealed that mutant p53 is inherently labile, similar to its wild type (wt) counterpart. Consistently, in response to stress conditions, both wt and mutant p53 accumulate in cells. While wt p53 returns to basal level following recovery from stress, mutant p53 remains stable. In part, this can be explained in mutant p53-expressing cells by the lack of an auto-regulatory loop with Mdm2 and other negative regulators, which are pivotal for wt p53 regulation. Further, additional protective mechanisms are acquired by mutant p53, largely mediated by the co-chaperones and their paralogs, the stress-induced heat shock proteins. Consequently, mutant p53 is accumulated in cancer cells in response to chronic stress and this accumulation is critical for its oncogenic gain of functions (GOF). Building on the extensive knowledge regarding wt p53, the regulation of mutant p53 is unraveling. In this review, we describe the current understanding on the major levels at which mutant p53 is regulated. These include the regulation of p53 protein levels by microRNA and by enzymes controlling p53 proteasomal degradation.

Keywords: mutant p53, Mdm2, miRNA, proteasomal degradation, cancer

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***Correspondence:**
Ygal Haupt
ygal.haupt@petermac.org

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INTRODUCTION

Wild type (wt) p53 is a tumor suppressor, which plays a key role in the cellular stress response. Abrogating p53 function is a key event in human cancer, leading to deregulated cell cycle, genomic instability, resistance to stress signals, and ultimately leading to cancer development (1, 2). Dysfunction of p53 occurs in half the cases of cancers by direct mutations in the gene, whereas in the remainder, p53 becomes dysfunctional through a variety of regulatory breakdowns. Mutant p53 fails to emulate the transcriptional program executed by wt p53 to provide a robust response to stress.

Most p53 mutations are missense (hotspot mutations – R175, G245, R248, R249, R273, R282) and occur at its DNA-binding domain, which accounts for the improper DNA engagement and disruption of transcriptional activity. P53 mutants also gain new oncogenic functions including resistance to chemotherapies, enhanced cell growth, metabolism, and invasion [reviewed in Ref. (3, 4)].

Surprisingly, mutant p53, like its wt counterpart, is inherently labile (5–7). Sustained degradation of wt p53 in healthy cells protects them against potent cell growth inhibition, while stress provokes p53 accumulation and activation (8). Similarly, mutant p53 accumulates in response to stress (7).

Thus, both wt and mutant p53 need to accumulate in order to execute their respective functions: wt p53 suppresses cancer while mutant p53 promotes cancer through its GOFs. It is therefore of great clinical importance to understand how wt and mutant p53 are regulated if we are to tailor treatments according to p53 status. That is, either reactivating wt p53 expression and function or counteracting mutant p53. In this review, we will outline the major levels at which mutant p53 is regulated and discuss the major players. As most of the regulation appears to occur post-transcriptionally [reviewed in Ref. (9)], this will form the major focus of this review.

REGULATION OF MUTANT p53 BY microRNA

MicroRNAs (miRNA) are the best-characterized members of the non-coding RNAs (ncRNAs) family. Typically these are 18–24 nucleotide (nt) RNA molecules that are not translated into proteins, and target messenger RNA (mRNA) species, through engagement of as few as six complementary nucleotides (10). Canonically, mature miRNAs bind mRNA 3′-untranslated-regions (3′-UTRs) and promote either target degradation or translational inhibition. Through engagement of coding regions (11), 5′-UTR (12), and open reading frames (ORF) (13), miRNAs can also regulate translation. The targeting flexibility of miRNAs allows them to affect multiple targets that are pertinent to both tumor suppression and oncogenesis: gene expression, protein regulation, homeostasis, and diseases.

The expression of both wt and mutant p53 are subject to miRNA regulation directly. MiRNAs targeting *p53* mRNA are incapable of discriminating between its wt and mutant mRNAs, unless an miRNA directly targets a mutated site. MiR-125b was the first miRNA demonstrated to bind *p53* 3′-UTR mRNA causing down-regulation of p53 protein and a consequent reduction in its activity, in human neuroblastoma cells and primary human lung fibroblasts (14). Additional p53-directed miRNAs have been identified both experimentally and from *in silico* analyses (**Figure 1**).

MicroRNAs also regulate p53 protein stability indirectly, by targeting its key regulators. For instance, the E3 ubiquitin ligase Mdm2, which is the major negative regulator of p53 [reviewed in Ref. (8, 15)] is extensively targeted by miRNAs for degradation. As a consequence of Mdm2 targeting, p53 (wt or mutant) is released from Mdm2-mediated ubiquitination and subsequent proteasomal degradation (**Figure 1**). Mdm4 (also known as MdmX), which is an Mdm2-related protein, is another key inhibitor of p53 transcriptional activity (16, 17) and is also targeted by miRNAs (**Figure 1**). Although miRNAs predominantly degrade mRNAs, an exceptional instance is miR-885-3p, which engages the 5′-UTR of Mdm4 mRNA and elevates Mdm4 protein levels (12).

Apart from Mdm2 and Mdm4, miRNAs also stabilize p53 through other regulatory pathways (**Figure 1**). MiR-29 activates p53 by targeting p85-alpha and CDC42 (18), miR-449 targets SIRT1 and HDAC1 (19), and miR-32 targets TSC1 and activates mTOR in human glioblastoma multiforme (20), all of which lead to the stabilization of p53. Recent study by Wang et al. demonstrated that miR 542-3p directly targets RPS23, resulting in subsequent RPL11 up-regulation, inhibiting Mdm2 and ultimately reducing proteasomal degradation of p53 (21).

Significantly for cancer, interaction of miRNAs with wt and mutant p53 is not unidirectional, and expression levels and biogenesis of miRNAs are affected by p53. Suzuki et al. demonstrated that wt p53 enhances post-transcriptional maturation of miR-16-1, miR-143, and miR-145 in response to DNA damage, while mutant p53 attenuates miRNA processing (22). Muller et al. further demonstrated that mutant p53 modulates miRNA processing, through direct inhibition of TAp63-mediated transcriptional activation of Dicer, and also through a TAp63-independent manner (23). Apart from global modulation of miRNA biogenesis, mutant p53 also affects expression of miRNAs, principally by downregulating tumor-suppressive miRNAs – miR-130b in endometrial cancer (24), miR-27a in breast cancer cells (MDA-MB-468) (25), miR-223 in breast and colon cells (26), let-7i in breast cancer and DLD1 cells (colorectal cancer) (27), and miR-205 (28), and elevating oncogenic miRNAs: miR-128-2 (29) and miR-155 in breast cancer cells (30) to mediate its oncogenic functions.

These studies collectively suggest that intertwined regulation of miRNAs, wt, and mutant p53 is vital to cancer. Given the importance of ncRNA in the regulation of wt and mutant p53, ncRNA represents feasible therapeutic targets for the development of new approach targeting mutant p53 in cancer.

REGULATION OF MUTANT p53 PROTEIN STABILITY

Overall, wt p53 is predominantly regulated at the protein stability level, under normal and stress conditions. Extensive study has defined that p53 stability is dictated by a variety of stabilizing post-translational modifications (PTM), while its degradation is largely the consequence of ubiquitination, executed by several E3 ligases [reviewed in Ref. (31, 32)]. Despite a drastic difference between the stability of mutant versus wt p53 in cancer cells, the majority of the regulatory pathways of p53 are shared between wt and mutant p53. However, a number of key differences promote the chronic stabilization and activation of mutant p53, which drive its oncogenic GOF. Understanding the regulation of mutant p53 has direct clinical implications. In this section, we will cover the major levels of mutant p53 regulation, with a focus on the degradation of mutant p53.

REGULATION OF MUTANT p53 DEGRADATION

p53 stability is tightly controlled by ubiquitin E3 ligases, which together with the enzymatic activities of E1 and E2, and in certain cases also E4 ligases coordinate the efficient degradation of proteins through the 26S proteasome machinery (33). The temporal and spatial modulation of the degradation of p53 is achieved by PTMs described below. In this section, we will outline the major E3 ligases that have been shown to control mutant p53 stability. A paradigm shift in our understanding of mutant p53 stability

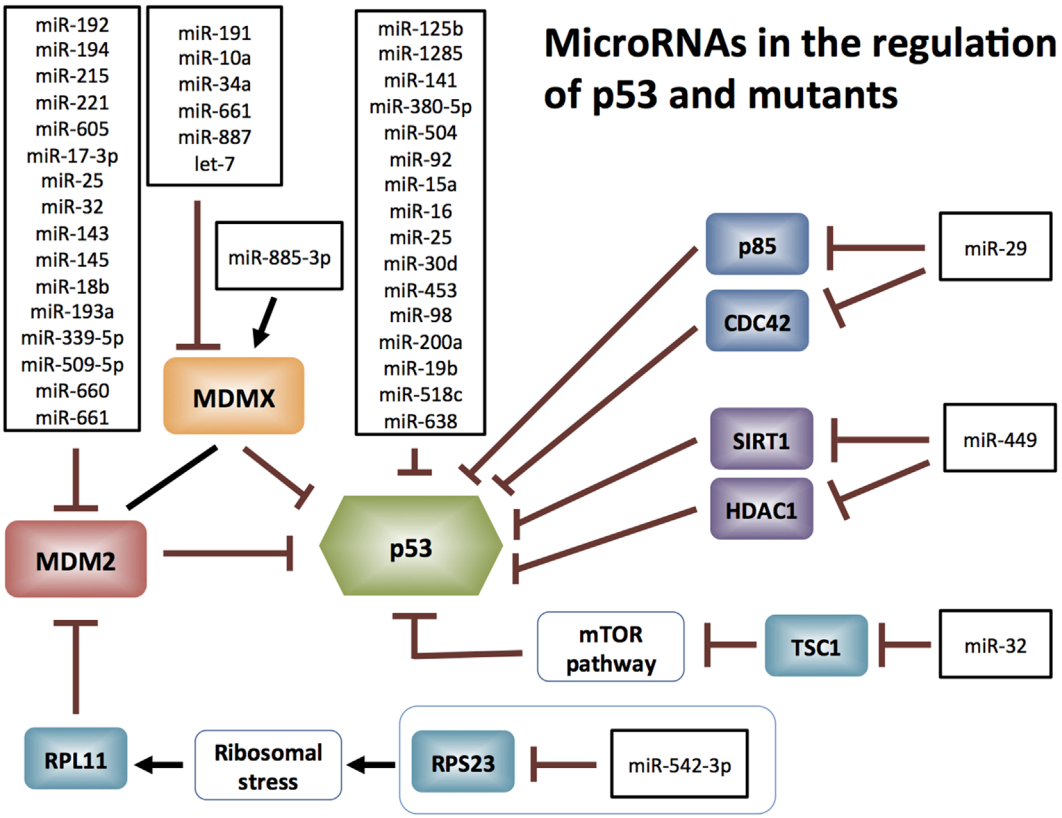

FIGURE 1 | MicroRNAs targeting p53: miR-125b, miR-504, miR-1285, miR-92, miR-141, miR-380-5p, miR-15a, miR-16, miR-25, miR-30d, miR-200a [reviewed in Ref. (88)**], miR-453** (89)**, miR-98** (89)**, miR-19b** (90)**, miR-518c** (91)**, and miR-638** (91). MicroRNAs targeting MDM2: miR-192, miR-194, miR-215, miR-221, miR-605, miR-17-3p, miR-193a, miR-25, miR-32, miR-143, miR-145, miR-18b, miR-661 [reviewed in Ref. (15)], miR-339-5p (92, 93), miR-660 (94), and miR-509-5p (95). MicroRNAs targeting MDM4: miR-191, miR-10a, miR-885-3p, miR-34a, miR-661 [reviewed in Ref. (15)], miR-887 (96), and let-7 (11).

was demonstrated in the studies of mutant p53 knock-in mice (5, 34). These papers showed for the first time that mutant p53 is inherently labile *in vivo*.

In the case of wt p53, the key physiological E3 ligase is Mdm2, which maintains p53 at low levels under basal conditions, and during recovery from stress (35, 36). Similarly, Mdm2 maintains the low basal levels of mutant p53 *in vivo* (7). In contrast to wt p53, mutant p53 does not form a feedback loop with Mdm2, as it is incapable of inducing Mdm2 transcription (37). Therefore, following stress-induced stabilization of wt and mutant p53, only wt p53 recovers to basal levels under the influence of Mdm2 (**Figure 2**). This can be corrected by enforced expression of Mdm2, which is able to efficiently degrade of mutant p53 (35). Mdm2 interacts with multiple domains of p53, which allows it to bind even to conformational p53 mutants, which are missense p53 mutations that either locally or globally disrupt the structure of p53, as distinct from "DNA contact mutants" (38, 39). The overall efficiency of mutant p53 ubiquitination, however, is reduced compared with that of wt p53 (40). The ubiquitination of mutant p53 is also enhanced by the activity of other E3 ligases: CHIP and Cop1 (40). Although ubiquitination of p53 by Mdm2 is enhanced by ubiquitin-interacting protein, hHR23a, the consequent ubiquitinated p53 accumulates but is not degraded (41). In a series of key *in vivo* studies, Terzian et al. (7) and Suh et al. (20) demonstrated that stress conditions (including oxidative stress, DNA damage) and oncogenic stress (such as the loss of p16) promote stabilization of mutant p53 and contribute to tumorigenesis. Subsequent studies have shown that multiple oncogenic effects can stabilize mutant p53 *in vivo* and drive its oncogenic functions. Interestingly, at least in the case of PML loss, the impact on mutant p53 GOF is gender-specific (42).

Degradation of mutant p53 has also been described to occur via alternative forms of autophagy. The first is: "Macroautophagy," which is triggered in response to starvation to recycle cellular contents through the lysosomes. A specific form of starvation is glucose restriction, which increases mutant p53 deacetylation, and sends it to degrade through the autophagic machinery. This degradation is Mdm2-dependent (43), but does not involve the proteasome (43, 44). A second form is: selective "chaperone-mediated autophagy" (CMA), in which specific cytosolic proteins are engaged by heat shock proteins (HSPs) and targeted to lysosomes. CMA is a normal cellular process that becomes more active in response to nutrient deprivation (43–46). Although mechanisms like autophagy seem to be less

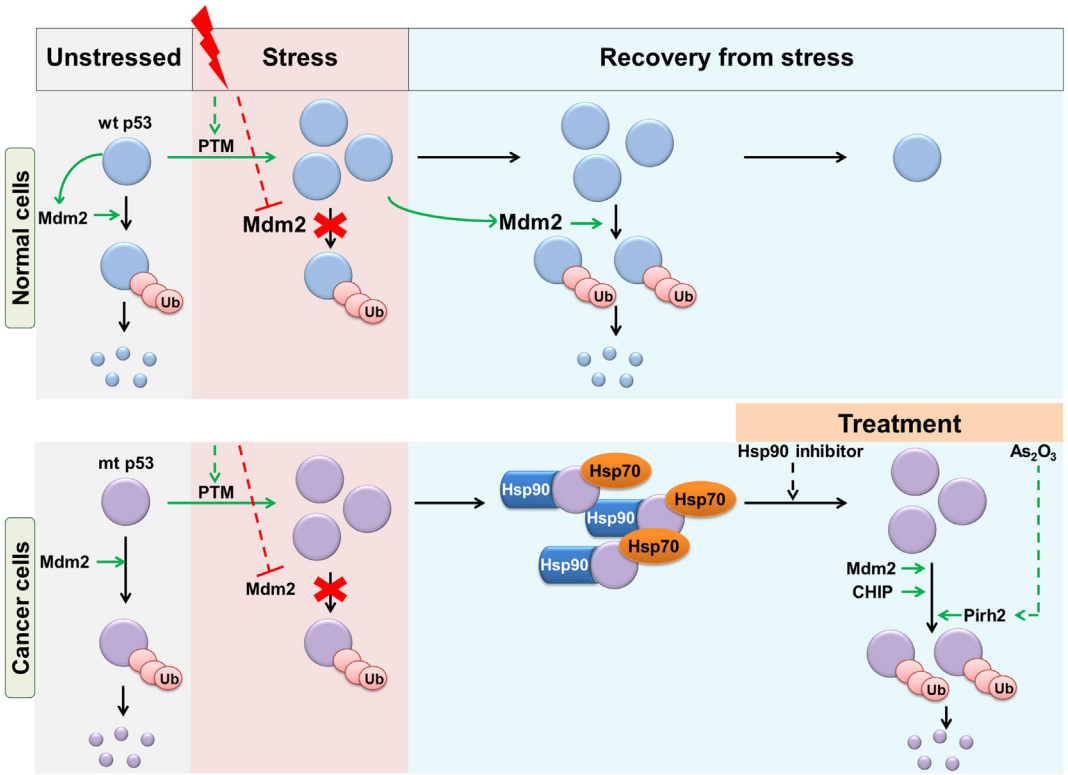

FIGURE 2 | Regulation of wt and mutant p53 stability in response to stress. Wt and mutant p53 are maintained at basal levels by Mdm2-mediated degradation. Upon stress stimulus, events such as post-translational modifications (PTM) result in stabilization and accumulation of p53. During recovery from stress, wt p53 returns to basal levels as a result of negative feedback through Mdm2. However, mutant p53 accumulates due to its failure to transactivate Mdm2. Mutant p53 is also protected from Mdm2 and CHIP mediated ubiquitination by chaperone proteins. This can be overcome by treatment with Hsp90 inhibitors. Treatment of cells with As_2O_3 induces the E3 ligase Pirh2, which ubiquitinates mutant p53.

specific than proteasomal degradation, it has been observed that ubiquitinated proteins are targeted for lysosomal degradation and could play a major role in regulating mutant p53 (47).

CHIP is a chaperone-dependent E3 ligase able to ubiquitinate misfolded proteins, a process which is assisted by the Hsp70/90 chaperone machinery. Hsp90-bound substrates are protected from ubiquitination, whereas Hsp70-bound substrates are ubiquitinated by CHIP (48). Interestingly, Hsp70 is found to partially inhibit Mdm2-mediated degradation of mutant p53. This apparent discrepancy in Hsp70 activity with respect to the two E3 ligases is not completely understood. In contrast, Hsp90 protects mutant p53 from both CHIP and Mdm2-mediated degradation (49, 50). This is because mutant p53, unlike wt p53, forms a stable complex with Hsp90 (51, 52). Inhibition of Hsp90 by 17AAG or siRNA against HSF1 (a major transcription factor for Hsp) enhances the ubiquitination and degradation of mutant p53 (**Figure 2**) (53, 54). Indeed, it was observed that inhibition of Hsp90 reduced the viability of mutant p53-expressing cancer cells of breast (MBA-MB-468, MDA-MB-231, T47D), prostate (DU145), and colon (SW480) (49). Similarly, inhibition of HDAC6, a positive regulator of Hsp90, destabilizes mutant p53 and is preferentially toxic to mutant p53-expressing cancer cell lines (54, 55). Hsp90 directly, or indirectly via its transcriptional activator HSF1, is upregulated in many cancer types, which may contribute to mutant p53 stabilization (56). Mutant p53 exists in a positive forward loop with HSF1. Mutant p53 enhances HSF1 recruitment to DNA, thereby increasing the levels of HSPs, which further stabilize mutant p53 (57, 58). Indeed, treatment with Hsp90 inhibitor, 17DMAG (a derivative of 17AAG), can greatly reduce lymphoma formation and is known to improve survival in mice with mutant p53 (59). Lastly, RING domain containing E3 ligase, Pirh2, which directly ubiquitinates wt p53 (60), also interacts with and promotes the ubiquitination of mutant p53 (61, 62). Since Pirh2 is a p53 target gene, this negative feedback regulatory loop is interrupted by mutations in p53 (60). Treatment of mutant p53-expressing cells with Arsenic trioxide can induce Pirh2-mediated proteasomal degradation of mutant p53 (**Figure 2**) (62). The role of other E3 ligases, including ARF-BP1, which regulate wt p53, have been shown not to regulate mutant p53 [(40) and reviewed in Ref. (8)].

Countering the E3 ligases are the deubiquitinating enzymes (DUBs), which cleave ubiquitin from proteins. Unlike E3 ligases, the role of DUBs in controlling mutant p53 stability is poorly explored. USP10 deubiquitinates and stabilizes both

wt and mutant p53 (63). Inhibition of USP10 by the protein spautin-1 reduces mutant p53 levels under glucose-restricted conditions (46). The USP7 DUB has a complex interplay with both wt p53 and Mdm2, and it deubiquitinates the p53 activator, Tip60 (64). To date, no correlation between USP7 and p53 status has been identified in cancers (65, 66). In wt p53-expressing cell lines, inhibition of USP7 stabilizes p53 and promotes apoptosis. But in at least one mutant p53-expressing cell line, the inhibitor had no effect (67). ABRO1 is able to deubiquitinate wt p53 and stabilize it by facilitating its interaction with USP7. However, there is no information pertaining to its ability to deubiquitinate mutant p53. Overexpression of ABRO1 in HT29 (colorectal adenocarcinoma) and BT474 (breast cancer) cells (both expressing mutant p53) results in increased cell growth, which can be correlated with mutant p53 stability (68). Another DUB, UCHL1, stabilizes wt and mutant p53 levels in breast cancer cell lines and affects cell viability by a mechanism remaining to be explored (69). USP29 can also deubiquitinate p53 in response to oxidative stress (70).

Although much of the focus in the field has been devoted to the 26S-mediated proteasomal degradation of p53 during post-stress recovery, the 20S proteasome has been identified as the destination of unmodified p53 that is inherently unstable, unless protected by the NADH quinone oxidoreductase 1 (NQO1). Specifically, inhibition of NQO1 (for example by dicoumarol) promotes p53 degradation through the 20S proteasome in an Mdm2-independent manner. Interestingly, mutant p53 interacts strongly with NQO1, rendering it resistant to NQO1 inhibitors [reviewed in Ref. (71)]. Pertinently, NQO1 is elevated in many cancers, which may contribute to stabilization of mutant p53 in these cases [reviewed in Ref. (72)].

Free ribosomal proteins are known to regulate the Mdm2/MdmX-p53 axis and activate wt p53, thereby inhibiting tumor proliferation [reviewed in Ref. (73)]. For example, RPS27 is repressed by wt but not mutant p53, and increased expression of RPS27 stabilizes mutant p53 protein, thereby forming a feed forward loop in cancer (74). On the other hand, RPL26 not only binds to the 5′-UTR of p53 mRNA and enhances translation but also interacts with Mdm2 and protects p53 from degradation (75, 76).

POST-TRANSLATIONAL MODIFICATIONS OF MUTANT p53

The regulation of wt p53 degradation is modulated by PTMs. Wt p53 is extensively modified post-translationally in response to stress conditions, which lead to the stabilization and/or activation of p53 [reviewed in Ref. (31)]. Critically, in most of the tested cases, the PTMs of p53 are non-discriminatory between wt and mutant proteins (7, 31, 77). Some of these modifications contribute to mutant p53 stability by shielding it from degradation by Mdm2 (**Figure 2**). Specifically, phosphorylation of p53 on serine 20 and threonine 18 in response to DNA damage protects it from Mdm2 and leads to its activation and stabilization (77–79). These phosphorylations are also induced on mutant p53 in response to stress (49, 77). Mutant p53 also escapes from Mdm2 by constitutive phosphorylation by ERK (80). Similarly, activation of SIRT1, which deacetylates mutant p53, can reduce mutant p53 levels in triple-negative breast cancer cell lines, revealing the role of acetylation in stability of the mutant protein (81, 82).

In addition to mutant p53 itself, modifications of Mdm2/MdmX contribute to the protection of mutant p53 from these key inhibitors. In response to DNA damage, Mdm2 is phosphorylated by ATM and c-Abl, which compromises the ability of Mdm2 to degrade p53 (83, 84). ATM-mediated phosphorylation contributes also through the impaired oligomerization of Mdm2 (85). Similarly, MdmX, the key inhibitor of p53 is phosphorylated by ATM and c-Abl, which impairs its capacity to inhibit p53 (86, 87). To what extent these key phosphorylations affect mutant p53 is yet to be demonstrated. The indiscriminate modifications of wt versus mutant p53 in response to stress can contribute to mutant p53 accumulation and activation.

CONCLUDING REMARKS

While wt and mutant p53 have distinct and opposing effects on cancer cells, many aspects of their regulation are shared. The majority of the positive and negative regulators of wt p53 that have been tested have a similar regulatory effect on mutant p53. Critically, however, the tightly controlled myriad of positive and negative auto-regulatory loops, which govern wt p53 levels, is uncoupled in the context of mutant p53. In addition, mutant p53 is recognized as a misfolded protein by the heat shock protein chaperons. Together, these contribute to the protection of mutant p53 from the well-coordinated recovery from stress conditions. This results in the chronic accumulation of active mutant p53, which exerts its gain of functions. It is therefore of prime importance to screen patients for p53 mutations prior to treatments, which are known to activate and stabilize p53. The identification of mechanisms that protect mutant p53, as shown by the chaperon HSP proteins, identifies novel approaches to expose mutant p53 to its negative regulators and drive its destruction. Future studies identifying the unique protectors of mutant p53 are a rational approach to define novel approaches to target mutant p53 in cancer cells.

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RV contributed to writing and editing the paper and prepared a figure. KT contributed to writing and prepared a figure. PJM contributed to writing. SH and YH contributed to writing and editing the paper.

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Alterations in Mitochondrial and Endoplasmic Reticulum Signaling by p53 Mutants

*Carlotta Giorgi[1], Massimo Bonora[1], Sonia Missiroli[1], Claudia Morganti[1], Giampaolo Morciano[1], Mariusz R. Wieckowski[2] and Paolo Pinton[1]**

[1] Section of Pathology, Oncology and Experimental Biology, Laboratory for Technologies of Advanced Therapies (LTTA), Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy, [2] Department of Biochemistry, Nencki Institute of Experimental Biology, Warsaw, Poland

The p53 protein is probably the most important tumor suppressor, acting as a nuclear transcription factor primarily through the modulation of cell death. However, currently, it is well accepted that p53 can also exert important transcription-independent pro-cell death actions. Indeed, cytosolic localization of endogenous wild-type or transactivation-deficient p53 is necessary and sufficient for the induction of apoptosis and autophagy. Here, we present the extra-nuclear activities of p53 associated with the mitochondria and the endoplasmic reticulum, highlighting the activities of the p53 mutants on these compartments. These two intracellular organelles play crucial roles in the regulation of cell death, and it is now well established that they also represent sites where p53 can accumulate.

Keywords: calcium, mitochondria, endoplasmic reticulum, apoptosis, cell death

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****Correspondence:***
Paolo Pinton
pnp@unife.it

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INTRODUCTION

The p53 protein is the product of one of the most frequently mutated tumor suppressor genes in human cancer (TP53), playing a crucial role in the response of a myriad of intracellular pathways (1). Despite this master role, more than 50% of human cancers harbor somatic p53 gene mutations (2).

Unlike most tumor suppressor genes, which are predominantly inactivated by deletions or truncating mutations during cancer progression, the TP53 gene in human tumors often contains missense mutations that produce a full-length protein containing only a single amino acid substitution (called naturally occurring mutants) with a greatly prolonged half-life (3, 4).

Mutations in p53 result in both loss-of-function and gain-of-function activities (5–7).

In addition to its nuclear activity, there is much evidence to support the idea that the cytoplasmic pool of p53 plays a pivotal role in inducing apoptosis through a transactivation-independent mechanism (8) and **Table 1**.

The overexpression of a mutant p53, lacking most of the DNA-binding domain (DBD) and completely deficient in transactivation function, still triggers apoptosis (14). Moreover, the overexpression of a number of transactivation-incompetent p53 mutants efficiently induces apoptosis (16). Similarly, it was shown that p53 is able to trigger apoptosis even in the absence of a nucleus (13). However, these studies did not provide any mechanistic explanation; only several years later, a breakthrough study showed a direct role of p53 in mitochondrial apoptosis (17).

Mitochondria play a pivotal role in cell death (18), including apoptosis. In healthy cells, the inner mitochondrial membrane, the barrier between the intermembrane space (IMS) and the matrix, is nearly impermeable to all ions. Mitochondrial membrane permeabilization, e.g., through opening

TABLE 1 | p53 mutants localization and function.

P53 mutant	Localization	Ability to induce apoptosis	Transcription independent	Reference
L-p53	M	Yes	Yes	(9)
R273H	C	No		(10)
R175H	N, C	No		(11)
L194F	N, C	No		(10)
R280K	N, C	No		(10)
G245S	N, C	No		
R248W	N, C	No		(11)
R249S	N, C	No		(11)
R282W	N	No		(11)
L22Q	N	Yes	Yes	(12)
W23S	N	Yes	Yes	(12)
ΔNLS	C	Yes	Yes	(13)
dl214	N, C	Yes	Yes	(14)
N1–102	C	Yes	Yes	(13)
M246I	N	No		(15)

N, nucleus; M, mitochondria; C, cytosol.

of the permeability transition pore (PTP) (19), is frequently the decisive event that delineates the balance between survival and death (20, 21).

Proapoptotic signals resulting in outer mitochondrial membrane (OMM) permeabilization induce the release of IMS proteins that, once in the cytosol, activate different cell death-associated pathways (22).

How p53, once at the mitochondrion, induces OMM permeabilization to trigger the release of proapoptotic factors from the IMS is described in depth in the next section.

The endoplasmic reticulum (ER) can connect to and act synergistically with other membranous structures, including mitochondria, in particular at ER–mitochondria contact sites, also known as mitochondria-associated ER membranes (MAMs), which have a distance of approximately 10–25 nm between the ER and the mitochondria (23, 24). The close proximity of the ER and the OMM explains how proteins situated on the opposing membrane faces could interact and thus "tether" the two organelles (25, 26).

The ER and the mitochondria reciprocally transmit danger signals through physical contacts (24, 27). In particular, the ER–mitochondrial cross talk plays a key role in decoding Ca^{2+}-mediated apoptotic signals (28–32).

p53 AND THE MITOCHONDRIA

Moll and colleagues showed that death signals induce p53 stabilization and rapid translocation (30–60 min) to the mitochondria in primary, immortal, and transformed cells (9, 17, 33). The main evidence for a sequence-specific transactivation (SST)-independent pathway for p53-induced apoptosis comes from Mihara et al., who demonstrated that bypassing the nucleus by targeting p53 to the mitochondria (*via* fusion with a mitochondrial import leader peptide, designated L-p53) was sufficient to launch apoptosis in p53-deficient tumor cells (9) and to suppress colony growth, although not as strongly as nuclear p53.

When a portion of endogenous p53 trafficked to the mitochondria, it interacted with the Bcl-2 family members, including the anti-apoptotic Bcl-2 and Bcl-xL at the OMM to block their functions (9). Interestingly, through nuclear magnetic resonance (NMR) spectroscopy, it has been demonstrated that the p53 DBD is involved in this protein–protein interaction (34). Indeed, mutant p53-harboring breast cancer cells, such as MDA 468, SKBr3, T47D, and MDA 231 (missense p53 mutations R273H, R175H, L194F, and R280K, respectively, both structural and DNA-binding mutant types), were unable to elicit this interaction (34).

DNA-binding domain represents the central core domain of p53 structure required for sequence-specific DNA binding (residues 102–292), and it is the most highly conserved region where more than 80% of p53 mutations occur.

Moreover, trafficking wild-type (wt) p53 within the mitochondria displayed the activation of proapoptotic members, such as Bax and Bak (13, 35, 36), to induce their oligomerization by forming a pore in the OMM. As a result, the oligomerization allowed the release of cytochrome *c* into the cytoplasm, resulting in apoptosis induction (33). To gain functional insight, they were brought forward *in vitro* experiments using purified recombinant p53 proteins at increasing concentrations. Because modifications in the p53 DBD (represented by the p53 R175H and p53 R273H naturally occurring mutants) were strictly unable to promote cytochrome *c* release and the fact that p53 L194F, R280K, G245S, R248W, R249S, and R282W missense mutants destroyed any attempt to mediate OMM permeabilization (**Figure 1**), the integrity of the p53 DBD was declared essential for SST-independent apoptosis as well as for transcription-independent cell death.

In contrast, conflicting results can be seen in previously published papers, in which specific mutations that abolish the trans-activating functions of p53 (L22Q and W23S) in the amino-terminal domain (residues 1–42) or impair the p53 nuclear localization signal (p53 ΔNLS) in the C-terminal region (residues 324–393) did not fully abolish the p53 apoptogenic potential (14).

In 1995, Haupt and coworkers reported that a truncated p53 protein, containing only the first 214 residues of wt p53 (p53 dl214) and incapable of SST, appeared as a potent inducer of apoptosis. Similar experiments were conducted with a protein containing two point mutations (p53 Gln22Ser23), and in this case, the ability to induce efficient apoptotic cell death without SST properties, even though it occurred more slowly, was also confirmed (14). All of these sharply contradictory results culminated in the discovery of a p53 N1–102 truncation mutant as the minimal requirement for proapoptotic activity. This protein includes a mutated transactivation domain and an intact proline-rich region (residues 61–94 containing multiple copies of the PXXP sequence, where X is an amino acid) but lacks the central core and the C-ter previously mentioned. On the basis of these findings, p53 death signaling seems to depend on the proline-rich regulatory domain and does not require transactivation of target genes (13). Thus, as consequence, the DBD may be dispensable. The proline-rich region plays a role in p53 stability regulated by mouse double minute 2 homolog (MDM2). Indeed, p53 becomes more susceptible to degradation by MDM2 if this region is deleted (37).

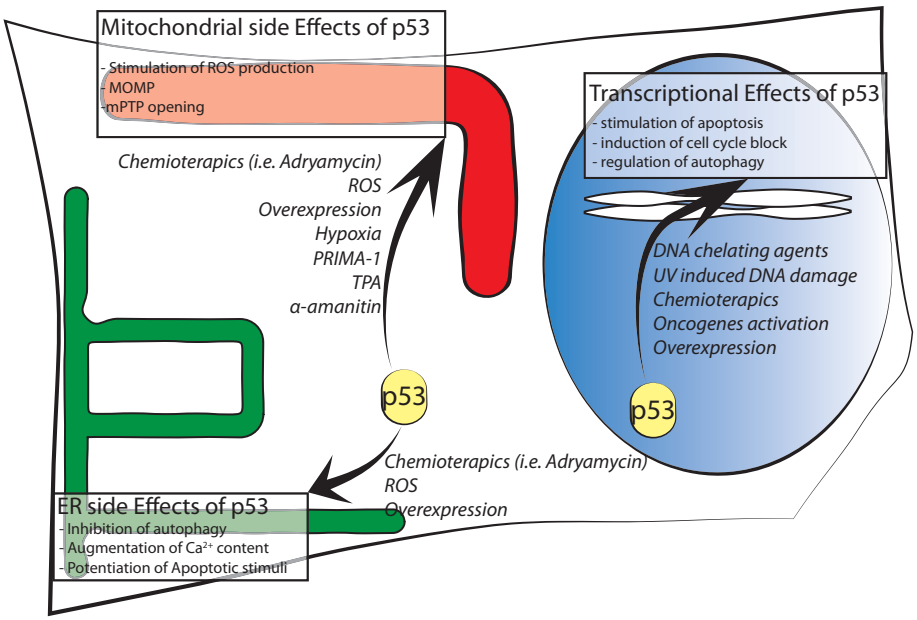

FIGURE 1 | **Schematic representation of p53 intracellular localizations known to date**. Major effects of intracellular distribution are shown in boxes while agents stimulating p53 localization are in italic font.

To be thorough, it should be considered that p53 mutants bind their interactors also in a DNA structure-selective mode as well as in a sequence-specific manner (38). However, this topic needs to be explored by additional studies.

Recently, it has been demonstrated how wt p53 can contribute to the apoptotic process through the caspase 3 protein. First, Sayan et al. demonstrated the presence of two caspase 3 cleavage sites in wt p53 at residues Asp^{21} and Asp^{186}. Because a portion of both proteins localized to the mitochondria, these authors compared the apoptotic activities of wt p53, non-cleavable mutants (D21A and D186A), and naturally occurring p53 mutants (R248W and R249S) (39). Their results, through overexpression experiments, suggested that following caspase activation p53 gains a transcription-independent function to reinforce apoptosis, leading to the formation of a positive feedback loop in which p53 accumulation induces caspase cleavage and promotes apoptosis. Later, Frank and coworkers identified and characterized pro-caspase-3 as a mitochondrial p53-interacting protein. This finding was detectable in wt p53 cells, upon stress induction and following Adriamycin treatment, but only 1–3% of the total wt p53 had this affinity. p53 R175H and R273H stably transfected cells shown how both the mutants were able to interact with caspase-3 at levels comparable to wt p53, but conversely impaired the ability of pro-caspase-3 to be activated by upstream caspases (40).

More recently, Sorrentino showed the strong requirement of the phosphorylation-specific peptidyl-prolyl cis/trans isomerase, NIMA-interacting 1 (Pin1) in the early phases of p53-dependent apoptosis by controlling its mitochondrial accumulation, activation, and increased retention (41). Phosphorylation at Ser46 on p53 by the homeodomain-interacting protein kinase 2 (HIPK2) is a key event in promoting its monoubiquitination and translocation to the mitochondria. These findings were intimately interconnected with the results previously obtained by Mihara and Mancini regarding the p53–Bcl-2 interaction and Ser46 phosphorylation of p53 (9, 42), and those results reported by Dumont et al. on the impact of the p53 codon 72 Pro/Arg polymorphism on the direct mitochondrial activity of p53 (37). In particular, the Arg72 displayed a higher level of translocation to the mitochondria, with this variant found to have higher phosphorylation on Ser46 and to be bound to Pin1 better than the Pro72 counterpart.

A small molecule known as PRIMA-1, which activates p53 to achieve tumor suppression by restoring the capacity of mutant protein to bind DNA, has been shown to induce the death of tumor cells expressing p53 and tumor-derived mutation p53 M246I and p53 R273H in the absence of transcription (13, 43).

Finally, another functional link between p53 and mitochondria originates from reactive oxygen species (ROS). ROS may contribute as actors on the stage of mitochondria–nuclear communication, interfering with the activity of various protein kinases and phosphatases (44, 45). Changes in mitochondrial function involving alterations in ROS production could affect p53 activity and its subcellular localization. In one specific case, p53 underwent an amino acid redox modification at cysteine 277 (oxidation of Cys277) in the p53 DBD that altered its DNA-binding affinity (46). Moreover, mitochondria-generated ROS induce p53 translocation to the mitochondria and in turn stimulate oxidative stress (46, 47) in part by transcription-independent mechanisms, leading to an increase in apoptosis. p53 translocates not only to the OMM but also into the matrix, where it was able to interact with protein–manganese superoxide dismutase (MnSOD, **Figure 1**),

leading to a reduction of its scavenging activity [Ref. (48) or Ref. (49) for an extensive review].

In the mitochondrial compartment, p53 has also been shown to accumulate following oxidative stress and ischemia, where it triggered PTP opening and necrosis (50). Thus, these results reveal a new role for p53 in activating necrotic cell death (**Figure 1**).

In the mitochondrial matrix also resides p53Ψ, an isoform product of an alternative 3′ splice-site activation of p53 mRNA. Consistent with previous information, this protein lacks SST functions and is sufficient to promote prometastatic features in epithelial cells (51). Moreover, cells expressing p53Ψ exhibited increased mitochondrial permeability and ROS production compared with cells that did not express this p53 isoform (51).

p53 AND THE ER AND MITOCHONDRIA-ASSOCIATED ER MEMBRANES

Another important subcellular localization of p53 is the ER, where it plays a critical role in the modulation of apoptosis and autophagy.

p53 regulates autophagy in a dual fashion: the pool of nuclear p53 stimulates autophagy in a transcription-dependent fashion (52, 53) and the pool of cytoplasmic p53 protein represses autophagy in a transcription-independent manner (54).

Suppression of autophagy is mediated by cytoplasmic, not nuclear p53. Indeed, Tasdemir at al. observed that p53 KO cells display higher autophagy levels compared to their wt counterpart. This effect was reverted by overexpression of wt p53, p53 with impaired nuclear localization sequence, and ER-targeted p53 (p53ER). A p53 form that was unable to exit the nucleus [using a disrupted nuclear export signal (NES)] failed to inhibit autophagy and the p53 inhibitor pifitrin α was able to induce autophagy in wt cells or nucleus deprived cells (54). Interestingly, the R175H mutation, which is known to inhibit the nuclear and cytoplasmic effects of p53 (34, 55), prevented inhibition of autophagy (54).

These results indicate that p53 inhibits autophagy through a transcription-independent effect exerted from a cytoplasmic localization. In fact, cytoplasmic p53 inhibits the AMP-dependent kinase, a positive regulator of autophagy, and activates mammalian target of rapamycin (mTOR), a negative regulator of autophagy. At present, the exact molecular pathway by which autophagy-inducing stimuli, such as ER stress, cause the cytoplasmic translocation and subsequent degradation of p53 remain unknown. It is known that its sequential phosphorylation of p53 on Ser 315 and Ser 376, the nuclear export, the ubiquitination by HDM2 and its proteasome-mediated degradation is required (56, 57). Inhibiting HDM2 or the proteasome prevents degradation of p53 induced by various autophagy triggers and inhibits autophagy.

In recent years, many other tumor suppressor proteins, such as PML and PTEN, have been demonstrated to localize to the ER and MAMs, where they regulate the ER–mitochondria Ca^{2+} flux and apoptosis (58, 59). p53 localization at the ER has been previously suggested (60), but this specific ER-localization was unable to regulate cell death induced by calcium-independent apoptotic stimuli.

We demonstrated that in the cytoplasm, wt p53 localizes at the ER and MAMs to modulate Ca^{2+}-mediated apoptosis in a transcription-independent manner (61). This non-nuclear fraction of p53 is able to modulate Ca^{2+} homeostasis in response to both physiological and pathological stimulation; in fact, activation and accumulation of p53 at the ER/MAMs render cells more prone to death, and the absence of p53 leads to lower steady-state levels of reticular Ca^{2+}, reduced Ca^{2+} mobilization, and mitochondrial accumulation evoked by agonist stimulation (ATP) or after the oxidative apoptotic inducer H_2O_2 (**Figure 1**). Importantly, to exclude the possibility that these effects are independent from the transcriptional activity of p53, different experimental strategies were used: a pharmacological inhibition of the transcriptional arm of p53 through the use of RNA polymerase II inhibitor α-amanitin (62), alone or in combination with pifitrin α, and the overexpression of p53 mutants lacking nuclear localization signal (p53 ΔNLS or ER p53).

Thus, p53 controls mitochondrial Ca^{2+} homeostasis and, in turn, apoptotic sensitivity from the ER/MAMs compartments.

Various naturally occurring p53 mutants, such as p53 R175H and p53 R273H, are unable to restore ER Ca^{2+} homeostasis when overexpressed in p53 KO cells, while the wt p53 efficiently does so. Accordingly, overexpression of wt p53, but not p53 R175H and p53 R273H, increased the sensitivity of p53 KO cells to oxidative stress back to the levels of their $p53^{+/+}$ counterparts, although there were no differences in the expression of apoptotic genes in cells expressing mutant p53. Moreover, cells harboring the p53 R273H mutation, such as MDA-MB 468 cells, did not display any significant alterations in ER Ca^{2+} levels when p53 was stabilized by adriamycin. Next, it was demonstrated that the Sarco/ER Ca^{2+}-ATPase Pump (SERCA), a pump responsible for maintaining high Ca^{2+} levels in the ER lumen (63), selectively binds wt p53 on the C-terminal portion, modulating its oxidation status; however, this domain alone is not sufficient to modulate Ca^{2+} homeostasis and apoptosis, indicating that this function requires the entire protein. Interestingly, the naturally occurring p53 mutants R175H and R273H were unable to bind SERCA and its oxidation was unchanged.

Taken together, all of these data suggest that Ca^{2+}-mediated apoptosis is a transcription-independent pathway regulated by p53 at ER/MAMs, through which p53 exerts its potent proapoptotic role in response to anticancer treatments.

To elucidate the relevance of these findings *in vivo*, we investigated the involvement of p53 in the control of intracellular Ca^{2+} signals and apoptosis in a 3D tumor mass in living mice (64). The use of a "skinfold chamber" installed on the back of athymic mice allowed the monitoring of tumor formation and, through a single-photon fluorescence microscope, the investigation of Ca^{2+} dynamics inside the tumor.

When detectable, the mass was stained with Fura-2, a Ca^{2+}-sensitive dye, and aluminum phtalocyanine chloride, a light-activated agent used in cancer photodynamic therapy (PDT). PDT accumulates in intracellular organelles, including ER and mitochondria, and after photo stimulation, it promotes Ca^{2+}-dependent apoptotic pathways. Through this technique, we demonstrated that p53 is able to modulate the Ca^{2+} response and that this is associated with reduced responsiveness to apoptotic

stimulation. Together, these results reveal a new mechanism by which p53 exerts its potent proapoptotic function in response to anticancer treatments.

Importantly, p53 can not only regulate cell survival through its activity at ER/MAMs sites but also alter ER functions that can control both localization and apoptotic activity of p53. It has been shown that ER stress inhibits p53-mediated apoptosis, modulating its localization and function (57). In fact, ER stress induces the cytoplasmic localization and enhances the destabilization of p53 due to phosphorylation at serine 315 and serine 376, which is mediated by glycogen synthase kinase-3-β (GSK3-β). As a result of the increased cytoplasmic localization, ER stress prevents p53 stabilization and p53-mediated apoptosis in response to DNA damage (57). Furthermore, it has been demonstrated that induction of the cytoplasmic translocation and degradation of p53 by ER stress is mediated by Hdm2 (56). This could have possible important implications for treatment of tumors with dysfunctional ER, aiming at p53 stabilization through the inhibition of the p53–Hdm2–GSK3-β pathway. Overall, these findings suggest that the cross talk of p53 with the dynamic ER plays a pivotal role in the regulation of cell survival and provides important evidence on how the specific targeting of the ER by tumor suppressors could counteract tumor progression.

FUTURE PERSPECTIVES

Despite the long-time knowledge on p53 involvement in tumorigenesis, its translation to clinical field has yet to be concluded. The studies summarized above clearly confirm that transcription-independent activities of p53 play an important role in the ability of the protein to activate several pathways in many circumstances. Nevertheless, many efforts still need to dissect the intricate signaling network that coordinates and couples the transcriptional and non-transcriptional proapoptotic activities of p53. In this way, there are still various mutants to be characterized, and how naturally occurring mutations affect p53 structure and function also remains elusive, as does the role of loss of wt and gain-of-function amino acid substitutions. Moreover, p53 extra-transcription activity studies using p53 mutants' overexpression should consider possible transcription activity alteration. For instance, high concentrations of p53 are demonstrated to inhibited p53-activated transcription by squelching (65, 66). Further studies should clarify how transcription and extra-transcription effects could cooperate.

This recognition and classification that not all p53 mutants are equivalent is important not merely as a conceptual distinction but may also have practical implications.

AUTHOR CONTRIBUTIONS

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

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Oncogenic Intra-p53 Family Member Interactions in Human Cancers

*Maria Ferraiuolo[1,2], Silvia Di Agostino[1], Giovanni Blandino[1] and Sabrina Strano[2]**

[1] Translational Oncogenomics Unit, Department of Molecular Medicine, Regina Elena National Cancer Institute, Rome, Italy,
[2] Molecular Chemoprevention Unit, Department of Molecular Medicine, Regina Elena National Cancer Institute, Rome, Italy

The p53 gene family members p53, p73, and p63 display several isoforms derived from the presence of internal promoters and alternative splicing events. They are structural homologs but hold peculiar functional properties. p53, p73, and p63 are tumor suppressor genes that promote differentiation, senescence, and apoptosis. p53, unlike p73 and p63, is frequently mutated in cancer often displaying oncogenic "gain of function" activities correlated with the induction of proliferation, invasion, chemoresistance, and genomic instability in cancer cells. These oncogenic functions are promoted either by the aberrant transcriptional cooperation of mutant p53 (mutp53) with transcription cofactors (e.g., NF-Y, E2F1, Vitamin D Receptor, Ets-1, NF-kB and YAP) or by the interaction with the p53 family members, p73 and p63, determining their functional inactivation. The instauration of these aberrant transcriptional networks leads to increased cell growth, low activation of DNA damage response pathways (DNA damage response and DNA double-strand breaks response), enhanced invasion, and high chemoresistance to different conventional chemotherapeutic treatments. Several studies have clearly shown that different cancers harboring mutant p53 proteins exhibit a poor prognosis when compared to those carrying wild-type p53 (wt-p53) protein. The interference of mutantp53/p73 and/or mutantp53/p63 interactions, thereby restoring p53, p73, and p63 tumor suppression functions, could be among the potential therapeutic strategies for the treatment of mutant p53 human cancers.

Keywords: p53 gene family members, gain of function, homology, isoforms, protein–protein interaction, target genes, apoptosis, differentiation

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****Correspondence:***
Sabrina Strano
strano@ifo.it

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INTRODUCTION

p53, p73, and p63 proteins belong to a family evolutionarily conserved in animals. They derive from an ancestral gene by duplication and consequent divergence of the original sequence. Functional and phylogenetic analyses reveal that the founding member was p63, followed by p73 and lastly p53 (1–4). In fact, at the sequence level, p63 and p73 display elevated homology to each other, more than to p53 (5–9). Generally, the protein structure consists of a central DNA-binding domain (DBD) (core domain) that binds to response elements of target genes (10–14). The N-terminal transcription-activation domain (TAD) is the binding-site for positive (e.g., p300/CBP and TAFII40/60) or negative regulators (e.g., MDM2 and MDMX) of gene transcription (15). The C-terminal oligomerization domain (OD) is subject to splicing and post-translational modifications, and it has been shown to influence DNA binding and transcriptional activity of the p53 family members (16) (**Figure 1**).

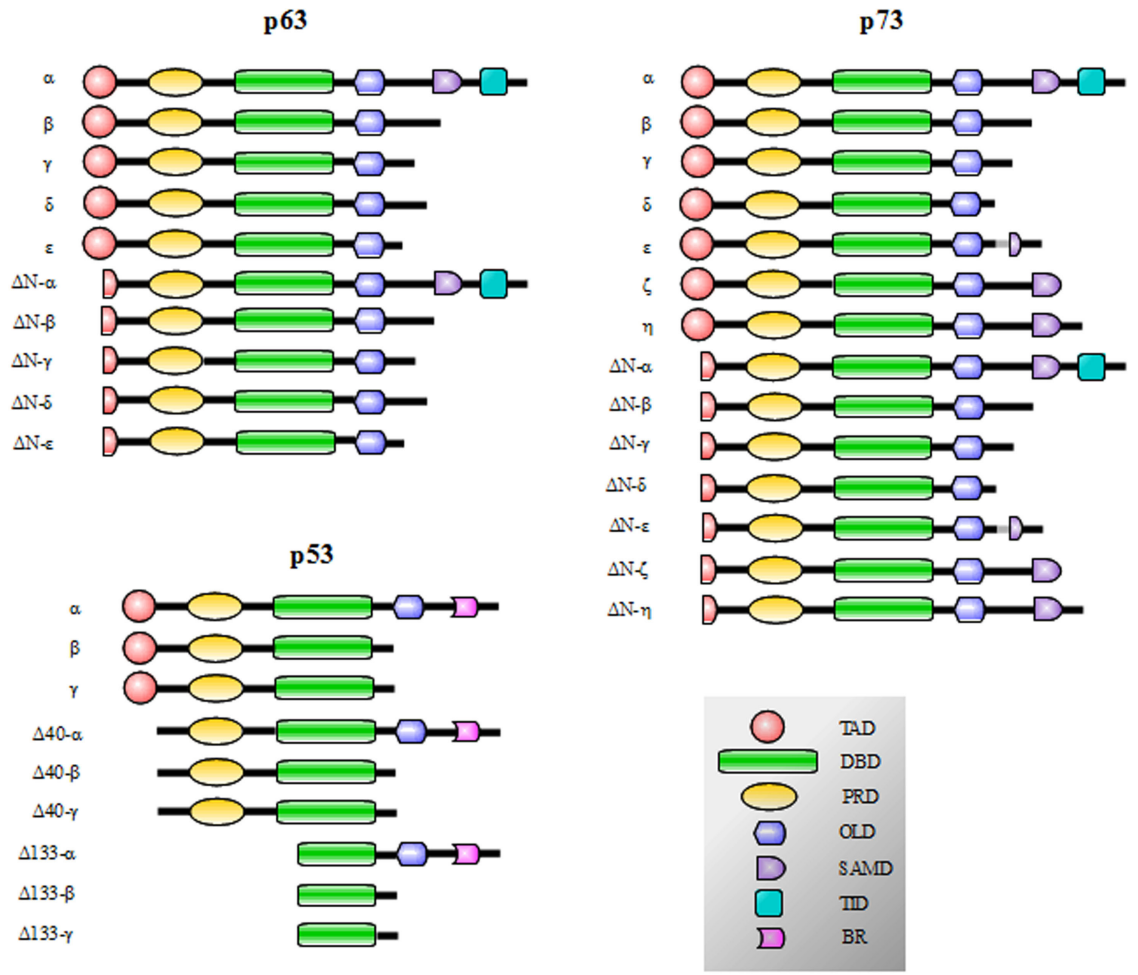

FIGURE 1 | p53, p73, and p63 isoforms obtained by the presence of internal promoters in the sequence (ΔN isoforms) and by alternative splicing. The N-terminal transcription-activation domain (TAD) contains two subdomains (AD1 and AD2) and is the binding-site for regulators of gene transcription; in p63 and p73 proteins, the C-terminal transactivation inhibitory domain (TID) binds to the TAD preventing constitutive transcriptional activity. The Proline-rich sequence recognition domains (PRD) can recognize proline-rich motifs of interacting proteins and has been reported to be essential for the induction of apoptosis driven by p53, p73 and p63. The DNA-binding domain (DBD) is responsible for the binding to DNA consensus of target genes and the oligomerization domain (OD) enables monomers assembly in active oligomers. The sterile alpha motif domain (SAMD) of p63 and p73 is arranged in a small five-helix bundle and is involved in protein–protein interactions. The basic region (BR) in the C-terminal of p53 is involved in the control of the DNA binding affinity.

The human *TP53* gene (Chr.17p13.1) is about 20 kb, contains 11 exons and encodes for the tumor suppressor p53 protein known as "the guardian of the genome" (17–28). This protein was discovered in 1979 (29–31). p53 is not only able to act as a transcription factor but it is also involved in transcription-independent response, such as apoptosis (32). *TP53* is the most frequently mutated gene in human cancers (33–36). It is mostly affected by missense mutations often in the DBD of the protein (37–39). An increasing number of p53-mutated proteins can be distinct in either conformational mutants (when the mutations change the tridimensional structure of the protein) or in DNA-contact defective mutants (when the mutations affect the region designate for the binding to the DNA) (36, 40). These alterations lead to the inability of mutant p53 proteins to fully recognize the DNA consensus sequence for wt-p53 or alter the functional interaction with pro-apoptotic partners, such as WW domain-containing oxidoreductase WOX1 (WWOX). The WWOX/wt-p53 complex is demonstrated to induce apoptosis synergistically and WWOX is essential for p53 activation and apoptosis induction (41). Strikingly, some of the p53-mutated proteins acquire new oncogenic functions [gain of function (GOF)] that strongly contribute to increasing cell proliferation, invasion, angiogenesis, genomic instability and chemoresistance in human cancers. These functions are often promoted by the interaction with sequence-specific transcription factors and the consequent activation/repression of specific target genes diverse from those recruited by wt-p53 (15, 42–58).

The *TP73* gene (Chr.1p36.33) is about 65 kb and contains 14 exons. It was discovered in 1997 (59) and similar to its family members, plays an important role at different regulatory

checkpoints of the cell cycle (60). However, p73 shows a peculiar function in neuronal differentiation, not shared with p53 (8, 61). p73 is rarely affected by mutation in cancer progression (62, 63) but its expression is deregulated in several human tumors, such as in hepatocellular carcinoma (64), neuroblastoma (65), lung cancer (62), prostate cancer (66) and colorectal carcinoma (67). p73 shares many p53 tumor suppression functions through the activation of the p53-target genes ($p21^{waf1}$, Bax, PUMA, NOXA, IGF-BP3 and Cyclin G), resulting in the control of cell proliferation, differentiation, development and induction of apoptosis (68–79). Moreover, p73, like p53, can interact with the tumor suppressor WWOX and trigger apoptosis (80, 81). Cells exposure to DNA-damaging agents (e.g., γ-radiation and cisplatin) induces p73 protein activation and accumulation with consequent induction of DNA damage response (DDR), growth arrest and apoptosis (76, 82–88).

The *TP63* gene (Chr.3q27–29) is approximately 65 kb and contains 15 exons (89, 90). Like p73, p63 can activate many p53-target genes in response to oncogenic stress or DNA damage (Bax, 14-3-3σ, p53AIP1, IGF-BP3, $p21^{waf1}$ and cyclin G), it controls cell proliferation, apoptosis, differentiation and development, and shows a tissue-specific localization (79, 91–94). p63 knockout mice exhibit a lethal phenotype soon after birth. They suffer from epithelial abnormalities, concerning skin, glands, teeth and hair follicles (95). Mutations of p63 are extremely rare in malignancies in contrast to p53 mutations. However, alterations in p63 expression patterns play an important role in tumorigenesis (96–98).

The full-length forms of p73 and p63 can also bind to YAP protein in response to DNA-damaging agents and activate pro-apoptotic target genes, such as Bax and p53AIP1 (78, 87, 99–101). Thus, both p73 and p63 can promote p53-independent apoptosis (102).

PROTEIN STRUCTURE AND RESPECTIVE ISOFORMS OF THE p53 FAMILY GENE MEMBERS

p53 family gene members show a high degree of similarity in the exon/intron organization and share a similar modular protein structure previously described (**Figure 1**). p73 and p63 proteins display 22–29% of homology in the TAD domain, 63% in the DBD, and 42% in the OD of p53. Furthermore, critical residues in the DBD, involved in the folding and binding to target DNA sequences, are strictly conserved (22, 103–106). Moreover, p73 and p63 share a sterile alpha motif domain (SAMD) and a transactivation inhibitory domain (TID); p53, p63, and p73 contain also a proline-rich sequence recognition domain (PRD). p53 also shows an additional basic region (BR) in the C-terminal tail (16, 107, 108) (**Figure 1**).

p53 protein displays nine isoforms obtained by the presence of cryptic internal promoters and by alternative splicing. The result is the presence of potentially transcript inert isoforms N-terminal deleted (ΔN) and with various C-terminal tails (108) (**Figure 1**). The same mechanisms occur for p73 and p63: p73 displays 14 isoforms and p63 exhibits 10 isoforms (59, 90, 109–117) (**Figure 1**). The N-terminal truncated isoforms ΔNp73 and ΔNp63 are highly expressed in the development and display an oncogenic dominant-negative function to p73 and p63 full-length (TAp73 and TAp63, respectively) and wt-p53 (39, 114, 118–124).

mutp53, p73, AND p63 PROTEIN INTERACTIONS IN CANCER

It was observed that human tumor-derived p53 mutants could bind p73α interfering with its transcriptional activity and impeding apoptosis induction (125, 126). Strano et al. (127) demonstrate that p53 mutants (p53R175H and p53D281G) associate with four p73 isoforms *in vitro* and *in vivo* (p73α, p73β, p73γ and p73δ). The interactions occur also in physiological conditions in breast cancer cell lines (T47D and SKBR3) and require the DBD of mutp53 and the DBD and OD domains of p73 isoforms. Marin et al. (126) show that the interaction between mutp53 and p73α or p73β is also governed by a polymorphism at the codon 72 of the p53 mutant proteins (e.g., mutp53R175H and mutp53V143A) that encode for Arginine (R) or Proline (P). Particularly, p53 mutants with R72 polymorphism favor binding to p73 more than the P72 polymorphism determining poor response to therapy and poor prognosis in patients (128, 129). Thus, either the type of p53 mutation and 72R/P polymorphism determine mutp53/p73 interaction (126). The mutp53/p63 interaction, *in vitro* and in tumor cells, is also reported (126). Gaiddon et al. (130) demonstrate that p73α, p73β, p73γ, and p73δ can interact with overexpressed or endogenous p53 mutants (R175H, H179R, Y220C, R248W and R273H) and demonstrate that p53 mutants (R175H, Y220C and R248W) can bind to p63α and p63γ. Low-affinity interactions are observed between mutp53R175H and ΔNp63α or ΔNp63γ. Moreover, they observe that the interaction between p73α and p63α or ΔNp63α is more efficient if p73α is mutated (R292H). Gaiddon et al. (130) confirm that p53 mutants require the DBD domain for the interaction with p73 or p63. Moreover, p53 mutants deleted in several regions, resulting in conformational changes of the DBD, are still able to bind p73 and p63. Thus, also the wild-type DBD of mutp53 can interact with p73 or p63 if it is in a mutant conformation (130, 131). It is also demonstrated that the heat shock protein HSC70 binds those p53 mutants that interact with p73 but not wt-p53 (130, 132), thus, other determinants could affect p53/p73 interaction (130). Strano et al. (46) demonstrate that, under physiological conditions, mutp53 interacts with p63α and p63γ in T47D and HaCat cells and in H1299 cells overexpressing mutp53R273H or mutp53R248W. They observe direct interactions mediated by the DBDs of mutp53 and p63. Mutp53D281G displays a GOF activity, it slightly binds to p73 but does not interact with p63 (46, 126, 127, 133). Moreover, mutp53D281G mutated in the TAD loses its GOF, suggesting that the TAD exerts an important oncogenic role in the GOF of this p53 mutant (15, 134, 135). Santini et al. (136) provide biochemical evidence on the interaction between mutp53R175H and p73. They use atomic force spectroscopy (AFS) (137, 138) and surface plasmons resonance (SPR) (139, 140), identifying a high interaction force and a dissociation equilibrium constant typical for specific bounds. They do not observe any interactions between wt-p53 and p73 (136), confirming the lack of *in vivo* evidence for the formation of wt-p53/p73 protein

complex. Weissmueller et al. (141) confirm that mutant p53 is able to bind p73 and this interaction results in the reduction of p73/NF-Y inhibitory complex in pancreatic ductal adenocarcinoma. This complex displays a tumor suppressor function repressing the oncogenic platelet-derived growth factor receptor b (PDGFRb) that promotes cell invasion and metastasis. Therefore, indirectly, mutant p53 promotes PDGFRb expression disassembling the inhibitory p73/NF-Y complex. Liu et al. (142) show that TopB1 protein promotes mutp53/NF-Y and mutp53/p73/p63 complex formation, inducing chemoresistance and proliferation in cancer cells. Wang and Fersht (143) describe the aggregation kinetics of mutant p53 that co-aggregate in tetramers by trapping also wt-p53, p73, and p63 proteins in the complex. It is worth to mention the role of MDM2 in the mutp53, p63, and p73 interplay: a recent work shows that MDM2, a negative regulator of wt-p53, competes with p63 for binding to mutp53R175H and in this way p63 activity is restored; but, on the other hand, MDM2 forms a trimeric complex with p73 and mutp53R273H strongly inhibiting p73 function (144). All these are clear examples of how different mutations in p53 protein could determine distinct protein–protein interactions and cell responses.

FUNCTIONAL IMPLICATION OF mutp53/p73/p63 PROTEIN INTERACTIONS IN CANCER

Knockout mice for $p53^{-/-}$, $p73^{-/-}$ and $p63^{-/-}$ highlight the major physiological roles of these proteins (96), suggesting pivotal functions in the development of nervous and immune systems (mediated by p73), in skin and limb development (mediated by p63) and in tumor suppression (mediated primarily by p53) (111, 145–148).

The role of p73 and p63 in tumorigenesis (p53-dependent or independent) is controversial. In many tumors, these proteins are downregulated, in others they are overexpressed or their genes are amplified. This apparent incongruence is mainly due to the different isoforms, tissue-specific localization and functions exerted by these proteins (121, 130, 149, 150). The decisive effect depends on the ratio TA/ΔN of p73 and p63 isoforms, p73/p63 interactions and p73/p63 binding to the promoters of p53-target genes. (151–155).

The status of p53 in cancer cells is a determining factor in the response to anticancer treatments (156–159). Some of the GOF activities mediated by mutp53 are related to the interaction, and consequent inactivation, of p73 and p63 (**Figure 2**). For example, the increase in chemoresistance to etoposide or cisplatin might involve the mutp53-dependent inactivation of p73-induced cell death (127, 128, 160–162). Importantly, in the presence of mutant p53 is observed a marked reduction of the transcriptional activity of p73α, p73β, p73γ and p73δ on the $p21^{waf1}$ promoter (127). Gaiddon et al. (130) show that the interaction between p73 and p53 mutants (R175H, Y220C and R283H) reduces p73 transactivation of the $p21^{waf1}$ promoter, highlighting the correlation between the capability of p53 mutants to interact with p73 and inhibit its transcriptional activity. Similar results are obtained for those p53 mutants that bind to p63α and p63γ reducing p63 activation of the $p21^{waf1}$ promoter (130). When mutp53 binds to the OD domain of p73, it causes the functional inhibition of

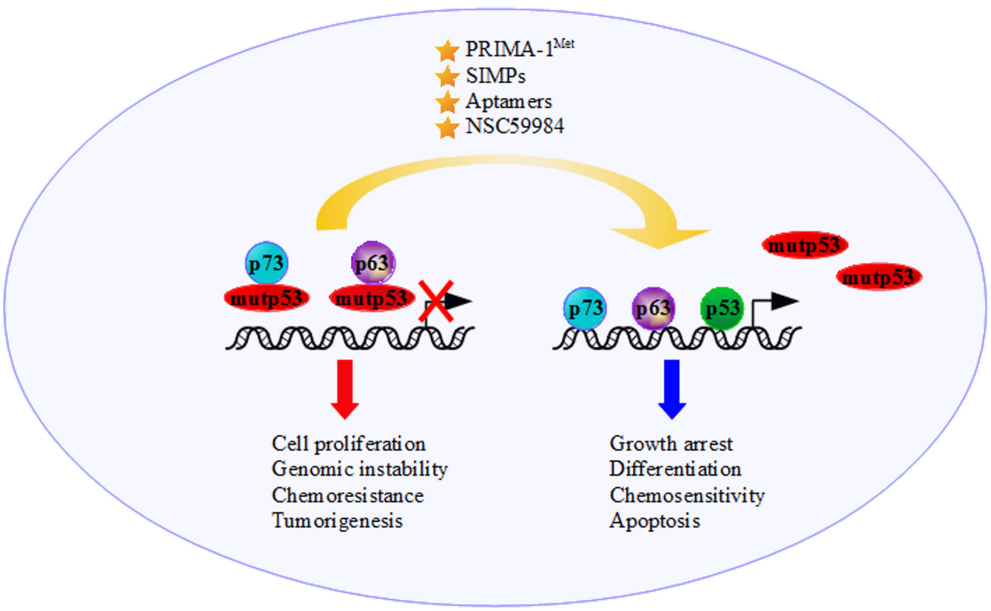

FIGURE 2 | The presence of mutant p53 moves the tumor suppressor functions of p73 and p63 to an oncogenic outcome through binding and inactivating p73 and p63 transcriptional activity. The development of new anticancer strategies, such as increasing p73/p63 activities and employing molecules that interfere with mutantp53/p73 and/or mutantp53/p63 interactions [e.g., the molecule p53 reactivation and induction of massive apoptosis (PRIMA-1^{Met}), short interfering mutp53 peptides (SIMPs), NSC59984 and Aptamers], could restore the p53 family's tumor suppressor functions.

p73, impairing the interactions with other modulators (162). Furthermore, when mutp53 binds to the DBD of p73, it provokes a physical sequester of p73 from the consensus sequences on the target genes (127, 163, 164). Similarly, mutp53/p63 interaction results in p63 impairment of the transcriptional activation of its target genes (Bax, $p21^{waf1}$, Cyclin G, 14-3-3σ and p53AIP1) (46). The formation of mutp53/p63 complex is also directly related to promoting cell invasion and metastasis in several cancer cell lines through mutp53-dependent inactivation of TAp63 tumor suppression functions (165–167).

In the past, many studies were dedicated to restoring the wild-type activity of the mutant p53 proteins (168). A great number of small molecules, aiming to restore and stabilize the original DBD conformation of p53, have been developed, such as p53 reactivation and induction of massive apoptosis (PRIMA-1) and maleimide-derived molecule MIRA-1. These compounds showed great promise when tested in cancer cell lines, demonstrating the induction of apoptotic processes (169–172). Unfortunately, the application of these molecules in the clinical practice is far off because the increased activity of p53 subjects non-cancerous cells to apoptosis induction. Further research is needed to minimize the level of cell toxicity (173). Another approach, proposed by Di Agostino et al. (174), refers to the disassembling of the mutp53/p73 complex using short interfering mutp53 peptides (SIMPs) (10–15 residues) that compete specifically with p73 for the binding of mutp53 to the DBD. This results in releasing p73 from the complex, activating apoptosis and rescuing cells from chemosensitivity. Notably, SIMPs have no cytotoxic effects on cells carrying wt-p53 proteins (174).

PRIMA-1^{MET} (APR-246, developed by APREA), a compound very similar to PRIMA-1 but much more active at low dosage, is discovered to restore mutant p53 (R273H and R175H) activity *in vitro* and *in vivo* (170, 175). Interestingly, PRIMA-1^{MET} not only restores the pro-apoptotic function of p53 but is also involved in activating downstream target genes of the p53 family (176, 177). PRIMA-1^{MET} alone and in combination with chemotherapeutic drugs are effective to induce apoptosis *in vivo* (178, 179). PRIMA-1^{MET} has also successfully completed a Phase I clinical trial, showing a promising efficacy (https://www.clinicaltrials.gov/ct2/show/NCT00900614). This molecule seems to lead to the formation of covalent adducts on mutant p53R175H and p53R273H proteins, but the exact mechanism of action has yet to be fully elucidated (180). Moreover, recently it is discovered that a small molecule, NSC59984, can restores wt-p53 signaling via p73 activation and induces p73-dependent cell death in colorectal cancer cells, without evident toxicity toward normal cells (181).

CONCLUSIONS

Flores et al. (164) demonstrated that wt-p53, p73 and p63 are recruited onto regulatory regions of the p53-target genes inducing growth arrest, differentiation, senescence and apoptosis (**Figure 2**). Despite this, the DBD of mutp53, previously regarded as "dead domain" since it could not bind to the wt-p53 binding site of its target genes, acquires a new protein–protein interaction function sequestering and inactivating tumor suppression proteins, including the family members p73 and p63. This mechanism contributes to the GOF activity of mutp53 (9).

FUTURE PERSPECTIVES

The precise tackling of GOF activity of mutant p53 might lead to the discovery of drugs with broad anticancer effects. As far as our knowledge on the molecular mechanisms governing mutant p53 oncogenic activities advances, we have learned that mutant p53 proteins are not a single entity but a protein family with high intrinsic complexities. Since mutant p53 is a partner of oncogenic multi-protein complexes, one way to severely defeat its pro-tumorigenic activity may reside in the specific targeting of its key cooperative partners. Along this line of evidence, agents that increase p73 and/or p63 activity promoting chemosensitivity could represent a promising strategy to treat tumors harboring mutant p53 proteins (122, 182, 183) (**Figure 2**). It could be useful to develop and validate reagents that interfere with mutp53/p73 and mutp53/p63 interactions restoring p53, p73, and p63 tumor suppression functions. NSC59984, PRIMA-1^{MET}, SIMPs and peptide aptamers, which bind specifically to mutant p53, could be a potent strategy in cancer therapy for these tumors (174, 181, 184) (**Figure 2**). Undoubtedly, greater knowledge must be acquired regarding the determinants of these oncogenic multi-protein complexes in order to design and pave novel therapeutic strategies to successfully treat mutant p53 human tumors.

AUTHOR CONTRIBUTIONS

The authors, MF and SDA, provided the structure and the writing of the article, which was integrated and supervised by SS and GB.

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REVIEW
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Mutant p53 Drives Cancer by Subverting Multiple Tumor Suppression Pathways

Sue Haupt[1,2], Dinesh Raghu[1,3] and Ygal Haupt[1,2,3,4]*

[1] Tumour Suppression Laboratory, Peter MacCallum Cancer Centre, Melbourne, VIC, Australia, [2] Department of Pathology, The University of Melbourne, Parkville, VIC, Australia, [3] Sir Peter MacCallum Department of Oncology, The University of Melbourne, Parkville, VIC, Australia, [4] Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC, Australia

The tumor suppressor p53 normally acts as a brake to halt damaged cells from perpetrating their genetic errors into future generations. If p53 is disrupted by mutation, it may not only lose these corrective powers, but counterproductively acquire new capacities that drive cancer. A newly emerging manner in which mutant p53 executes its cancer promoting functions is by harnessing key proteins, which normally partner with its wild type, tumor-inhibiting counterpart. In association with the subverted activities of these protein partners, mutant p53 is empowered to act across multiple fundamental cellular pathways (regulating cell division and metabolism) and corrupt them to become cancer promoting.

Keywords: p53 mutations, gain of function, metabolism, cell cycle, transcriptional regulation

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****Correspondence:***
Sue Haupt
sue.haupt@petermac.org

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INTRODUCTION

Reliance on the tumor suppressive capacity of p53 is profoundly emphasized by its near universal malfunction in all cancers. P53 is the most altered gene in cancer. More than 50% of human cancers are afflicted with a p53 mutation. Severe consequences of p53 mutation include the failure to protect against cancer stimuli, compounded by the acquisition of new cancer promoting, "neomorphic" properties, referred to as "Gain of function" (GOF), covered by other reviews in this series [reviewed in Ref. (1)].

A particularly sinister GOF constitutes the subversion by mutant p53, of molecular partners of wild type (wt) p53, and this strategy forms the focus of this review. Specifically, mutant p53 conscripts proteins that normally partner with wt p53. This new association divests them of their anticancer activities and in place, they are corrupted to act as promoters of tumorigenesis [e.g., Ref. (2)]. A number of fundamental cellular functions that are normally tumor suppressive under the directive of wt p53 become severely derailed under the influence of mutant p53 to promote cancer. Mutant p53 deregulates normally tightly controlled fundamental processes (including control of the mitotic cell cycle, glycolysis, nucleic acid, and lipid synthesis) to promote deregulated, proliferative cancer cell growth (**Figure 1**). Identifying the nature and the regulation of this mutant p53, GOF predicts therapeutic avenues for reining-in the impact of mutant p53 and fighting cancer.

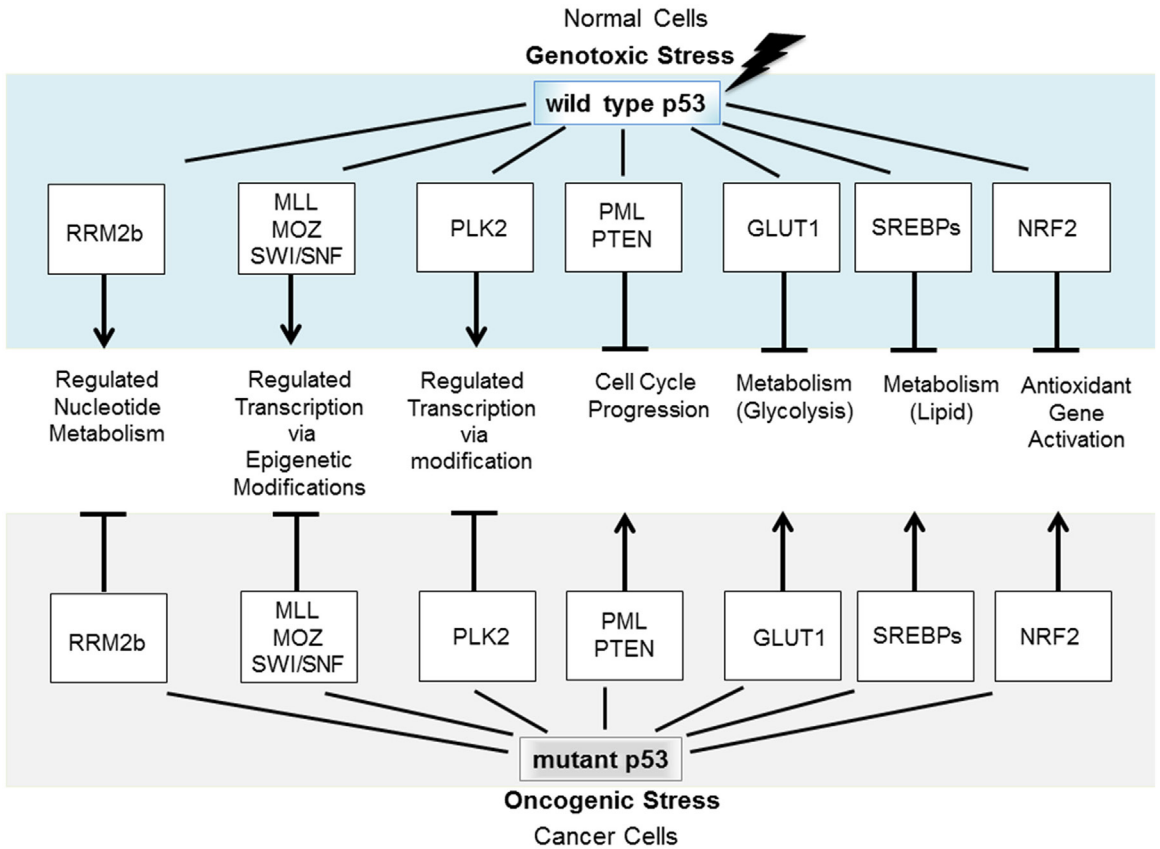

FIGURE 1 | Wt p53 is induced to accumulate in response to stress to regulate fundamental cellular processes that protect against tumorigenesis. If p53 becomes mutated, it not only loses these tumor-protecting capacities but also may gain new functions through coercion of partner molecular partners normally engaged by wt p53.

SUBVERSION OF CELL CYCLE REGULATION

Promyelocytic Leukemia

Proper cell cycle regulation is vital for normal cell function. Equally critical is the capacity to sense DNA damage and to interrupt the cycle to instigate repair or eliminate cells with irreparable damage, as appropriate. Wt p53 is a key dictator of cellular fate in response to DNA damage resulting from cellular stresses. Partnership with the tumor suppressor promyelocytic leukemia (PML) protein facilitates p53 stress responses. Specifically, wt p53 stabilization and activation in response to stress is promoted by PML, through temporal co-recruitment of post-translational modifiers of p53 [kinases: CK1 (3), CK2 (4), HIPK2 (5); acetylases: CBP/p300 (6); MOZ (7)], to functional service depots, known as "PML nuclear bodies" (PML-NBs). PML-NBs facilitate the addition of post-translational modifications to p53, which relieve it from its normally labile state. Stabilized wt p53 accumulates, halts cell cycle progression, and initiates molecular responses to either repair DNA or direct the execution of incurable cells. PML in turn is a direct target of wt p53 transcriptional activation, which defines a positive regulatory loop (8). Further, PML-NBs associate with sites of active transcription and appear to facilitate gene expression (9). PML loss alone does not cause cancer [at least in mice (10)]; however, interference with its function may promote cancer, as consistent with its discovery in acute PML, where PML is fused with RAR-alpha to generate the oncogenic PML–RAR-alpha (11).

Significantly, mutant p53 enslavement of PML defines a paradigm for mutant p53 disruption of tumor suppressive partners of wt p53. We identified that when p53 is mutated in cancer cells, its association with PML is constitutive, unlike the transient association with its wt p53 counterpart in response to stress. Importantly, PML facilitates mutant p53 to aberrantly transcribe targets in the context of hijacked transcription factor NF-Y [(2), building on foundational NF-Y studies (12)].

More explicitly, wt p53 is a transcription factor that regulates its target genes (to control DNA repair, growth, and metabolic cascades), through direct engagement of its responsive elements. In stark contrast, mutant p53 is unable to directly engage these specific elements, but rather anchors onto other transcription factors and interferes with their transcription [including NF-Y (12)]. One transcriptional target of mutant p53 in association with NF-Y and PML is CDC25C, which triggers entry into

mitosis (counteracting wt p53 activated growth arrest). Consistently, mutant p53 cancer cells may become growth dependent on PML, to the point where PML depletion leads to growth inhibition (2). Paradoxically, the capacity of PML to promote wt p53 as a tumor suppressor in healthy cells redefines PML as "oncogenic" when associated with mutant p53 in cancer cells [review in Ref. (13)].

At a higher level, cell cycle control is coordinated by the Circadian clock (14), and wt p53 defines a unique point of convergence between these two fundamental vital cellular regulatory systems. The Circadian clock is subject both to wt p53 (15) and PML (16) regulation and in turn regulates important cell cycle genes, including p21, independently of p53 (17) (**Figure 2**). While disruption of the diurnal periods of ~24 h appears insufficient alone to cause cancer, new findings suggest that it can exacerbate cancer progression [reviewed in Ref. (14)].

At a molecular level, the clock is comprised of at least nine interplaying proteins, and we will discuss only those pertinent to this review. The clock is positively activated in a cyclic fashion through the combined activities of the two transcription factors: CLOCK and BMAL1 (**Figure 2**). As heterodimers, they engage E-Box motifs in the promoters of their target genes and induce transcription. Important transcriptional target genes, Per and Cry, and their protein products relocate to the nucleus and negatively regulate CLOCK and BMAL1: forming a negative feedback loop. To restart the cycle, a stimulus such as light (or pertinently to our discussion DNA damage) must prompt elimination of Per and Cry, which is mediated through proteolysis [reviewed in Ref. (18)].

Wt p53 controls the clock through negative regulation of Per2 expression (**Figure 2**). Mechanistically, wt p53 competes for a promoter region of Per2 normally occupied by activating CLOCK/BMAL1 (15). In normal healthy cells, p53 levels oscillate temporally and Per2 levels inversely correspond. In cells undergoing stress, wt p53 accumulation inhibits Per2 transcription. On a background of mutant p53, cancer is exacerbated by mutation of either the clock regulatory gene Per2 (19), or PML loss (20). The capacity of PML to function as an upstream regulator of Per2 is consistent with a common regulatory pathway (16). In sum, interplaying regulatory loops between p53, PML, the circadian clock, and the cell cycle are emerging, and their disruption has been linked to cancer in mouse models (19, 20). Links to human cancers are also emerging, with the possibility of sleep hormone therapies being trialed [i.e., melatonin (21)].

Phosphatase and Tensin homolog

Phosphatase and Tensin homolog (PTEN) is also a vital cell cycle regulator that has achieved its reputation as a tumor suppressor in the context of wild type (wt) p53. Pten curbs cell cycle progression and cell survival by suppressing PI3K–AKT/PKB cell survival pathway (22). PTEN functions as a tumor suppressor by stabilizing p53 protein in an Mdm2-dependent and/or -independent mechanism. (23). PTEN also increases the transcriptional activity of wt p53 through physical interaction (24). Reciprocally, wt p53

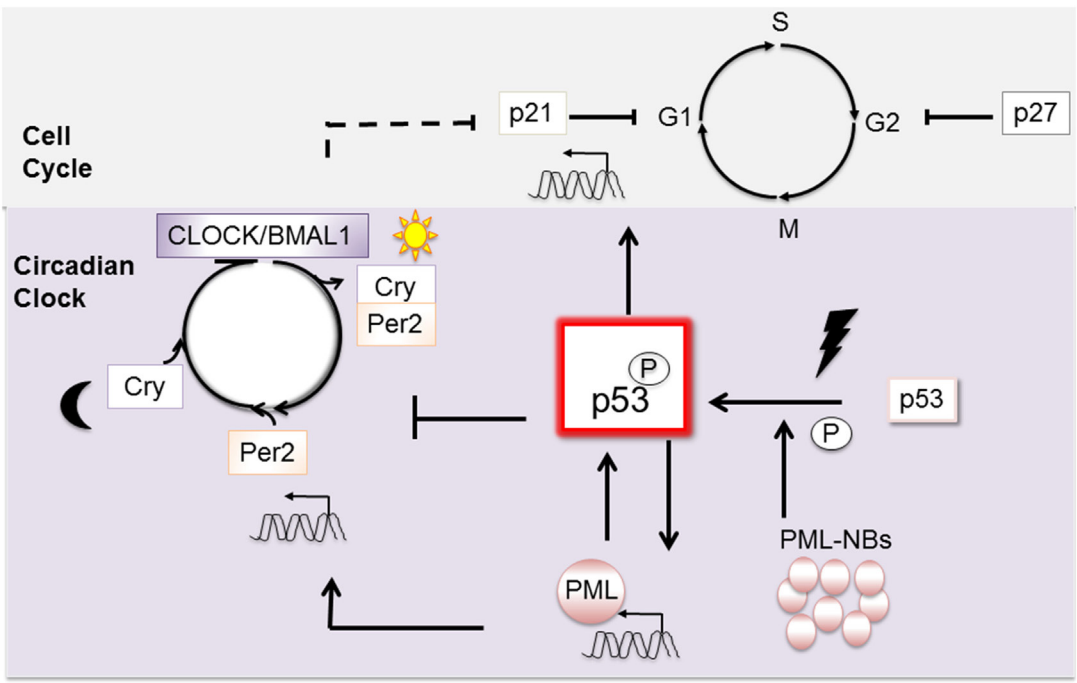

FIGURE 2 | Wt p53 is a pivotal point of connection between the mitotic cell cycle and the circadian clock. P53 activation is promoted by its transcriptional target PML. Once activated, wt p53 intervenes in the cell cycle through expression of its target gene, the checkpoint inhibitor p21. Upon stimulation, wt p53 can also intervene to affect the circadian clock. In contrast, when p53 is mutated, its interaction with PML becomes constitutive. Cancer is exacerbated when Per is mutated on a background of p53 mutation.

increases the transcription of PTEN by binding to the promoter of PTEN (25) and forming a feedback loop. These mutual relationships between PTEN and p53 promote tumor suppression.

In the context of mutant p53, in a diametrically opposing function, Pten promotes tumor growth (24). PTEN, in a comparable manner to PML, becomes oncogenic in cells expressing mutant p53 (26). PTEN stabilizes mutant p53 protein by inhibiting Mdm2-mediated degradation, which results in the inhibition of cell death and also in enhancement of cell proliferation (24). Additionally, PTEN increases the transcriptional activity of the mutant p53/acetylase CBP/NF-Y complex. This complex activates the transcription of c-Myc and Bcl-XL, which promotes cell survival and proliferation (26).

Polo-Like kinase-2

Polo-like kinase-2 (PLK2) is also a wt p53 target that contributes to cell cycle control. PLK2 is transcriptionally induced by wt p53 in response to the stress of DNA damage (27). PLK2 in a wt p53 setting is tumor suppressive, as engagement of p53 response elements in the promoter of PLK2 induces cell cycle arrest at the G2 checkpoint. In contrast, in a mutant p53 context, PLK2 functions as an oncogene. Distinct, indirect interaction between mutant p53 and PLK2, mediated through the conscription of the transcription factor NF-Y (to the CCAAT box promoter sequences), increases cell proliferation. A reinforcing feed back loop is created by PLK2 in turn phosphorylating mutant p53 on a site not phosphorylated on wt p53. Phosphorylated mutant p53 interacts more efficiently with p300 and promotes transcriptional activities of cell cycle activators (28). This feedback loop involving PLK2 defines a prototype cycle of reinforcement of mutant p53 GOF (29).

DIVERSION OF FUNDAMENTAL CELLULAR PATHWAYS

Rapid cell proliferation inherent in cancer growth is utterly dependent on the ready supply of "molecular building blocks." Recent studies have identified that fundamental metabolic processes normally regulated by wt p53 are extensively disrupted by mutant p53 to facilitate the supply of these necessities.

Nucleotide Metabolism

RRM2b

Proper repair of DNA damage is orchestrated by wt p53, which not only temporally halts cell cycle progression to allow repair, but also actively facilitates the supply of constituents for the repair. Specifically, in response to DNA damage, wt p53 transcriptionally activates the small subunit of the ribonucleotide reductase (RRM2b) in a temporary manner, to facilitate the catalytic conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, which is an essential step for DNA synthesis. In contrast, when p53 is mutated, it constitutively upregulates RRM2b expression. Importantly, the mechanism of transcriptional activation of RRM2b is dependent on the status of p53: where wt p53 engages its REs in the intronic region and in contrast mutant p53 localizes to the promoter. Further, it has emerged that mutant p53 transcriptionally drives additional nucleotide metabolic genes, both in the salvage and new synthesis pathways, through co-recruitment with the transcription factor ETS2, to its target gene promoters. ETS2 engagement by mutant p53 is a recurring theme, as we discuss below for epigenetic regulation. Overall, mutant p53 upregulates nucleotide biosynthesis, which contributes to meeting the voracious demands of rapidly proliferating and invading cancers (30).

Glucose Metabolism

Glucose Transporter 1

Regulated glucose metabolism is vital for maintaining healthy, normal cell homeostasis, in contrast to the voracious consumption of glucose that feeds cancer cell proliferation and is inherent in the "Warburg effect." Proper glucose regulation is then an important tumor suppressive capacity of wt p53. Wt p53 regulates glucose metabolism by restricting cellular glucose at three levels through (31): (1) suppression of the expression of glucose transporter 1 (GLUT1) and 4 (32); (2) transcriptional regulation of target genes, which inhibit glycolysis [TIGAR (33)] and gluconeogenesis in the liver (34); and (3) direct binding and inhibition of the rate-limiting enzyme (glucose-6-phophate dehydrogenase) in an alternative anabolic pathway [the pentose phosphate pathway (35)].

Profoundly, when p53 is mutated, not only are these points of regulating glucose metabolism lost but further glucose uptake is accentuated through a novel GOF. This disastrous mutant p53 GOF is the shunting of the glucose transporter, Glut1, to the cell membrane surface where it stokes glucose uptake by cancer cells (36). Elevated glucose levels feed into metabolic anabolism to provide the increased demand for the molecular building blocks required to support rapid cancer cell proliferation, inherent in the Warburg effect. Reciprocally, glucose maintains mutant p53 stability and promotes cancer cell growth (37), generating a positive regulatory loop.

Reliance on a mutant p53-dependent enhanced supply of glucose to foster cell proliferation defines a unique point of vulnerability in cancer cells. This appetite for glucose identifies a potential therapy target which is currently being extensively investigated [i.e., ketogenic diets (38) and repurposing of the widely used diabetic metformin (39)].

Lipid Metabolism

Sterol Regulatory Element-Binding Proteins

A controlled supply of lipids is vital for regulated cell division and maintenance. Nearly every enzyme in the fatty acid and cholesterol synthesis are subject to regulation by the transcription factor of sterol regulatory element-binding proteins [SREBPs (40)]. Specifically, SREBP-1 dictates expression of lipogenic enzymes including fatty acid synthase, while SREBP-2 regulates cholesterol synthesis [reviewed in Ref. (41)]. In response to stress, consistent with halting cell division, wt p53 restrains lipid accumulation by inhibiting expression of the transcription factor SREBP-1, and in turn triglyceride synthesis, and lipogenic genes (41). In contrast, mutant p53 engages the SREBPs (both SREBP-1 and -2) directly. Mutant p53 is recruited to SREBP target gene promoters

(although co-recruitment remains to be directly demonstrated). Mutant p53 appears to upregulate transcription of key enzymes in the sterol pathway and fatty acid biosynthesis pathway. Mutant p53 correlates with increased expression of enzymes in both the mevalonate synthesis (cholesterol) pathway and fatty acid synthesis pathways. Mutant p53 upregulation of these vital pathways is consistent with meeting increased demand for membrane lipids in rapidly proliferating cancer cells (42).

Antioxidant Pathways

Nuclear Factor Erythroid-Related Factor-2

Reactive oxygen intermediates perform important cellular functions including signaling; however, they are seriously damaging to normal cells if not properly contained and are linked to cancer [review in Ref (43)]. A master redox regulator is the transcription factor, nuclear factor erythroid-related factor-2 (NRF2) (44). P53 acts as a stress-rheostat controller of Nrf2 levels. Specifically, in response to mild stress, p53 transcriptionally activates the vital cell cycle inhibitor, p21, which binds to Nrf2 and consequently relieves it from its normal restraint (45). Relocation of NRF2 from the cytoplasm to the nucleus permits it to regulate multiple antioxidant targets, where some ~200 genes have been reported (44). These include the NADH-quinone oxidoreductase1 (NQO1), which also has differential function in a wt (46) versus mutant p53 context (47) (but will not be further elaborated here). When stress insults are severe, however, p53 inhibits Nrf2 (45). This exquisite level of control is consistent with p53 instigating repair in response to mild stress insults while intervening to prevent remedial action in those that are irrevocably damaged.

A novel GOF of mutant p53 is its capacity to reduce Nrf2 protein levels (without impacting its mRNA), in response to oxidative stress. The consequence is low levels of Nrf2 target detoxifying genes and elevated levels of reactive oxygen species (ROS). Remarkably, in contrast to growth inhibition imposed on wt p53 cells subject to oxidative stress, those with mutant p53 tolerate elevated ROS, survive, and proliferate (48).

INTERFERENCE WITH TRANSCRIPTIONAL REGULATION

When p53 is mutated, a radical shift in transcriptional activity occurs, which is conducive to cancer promotion. An altered repertoire of transcription factor engagement is emerging for mutant p53. While mutant p53 is not able to directly engage wt p53 response elements, it may instead directly bind its wt counterpart and impose a dominant negative effect over wt p53 functions, including depriving it of capacity to regulate transcription. Mutant p53 may also engage transcription factors that wt p53 does not, including the family members p63 and p73 and disrupt their functions. More specifically, the presence of arginine at codon 72 dictates the capacity of mutant p53 to sequester p73, where mutants with proline are incapable of this inactivation (49, 50).

Beyond this negative regulation of wt p53 and its family members, mutant p53 may hijack transcription factor partners and disrupt their normal transcriptional activity (as mentioned above). Mutant p53 has been reported to engage NF-Y, NF-kappa B, SP1, E2F1, ETS1, ETS2, and SREBP. The outcome may be altered target engagement, or a change in the rate of transcription relative to a wt p53 context. These features of mutant p53 have been comprehensively reviewed recently (51), so we will concentrate on new findings.

SWI/SNF

At a higher level, mutant p53 disruption of chromatin regulation is also now emerging. In order for wt p53 to access specific DNA responsive elements in the regulatory regions (upstream promoters or introns) of its target genes, it must coordinate with numerous chromatin regulators to expose appropriate regulatory elements and associated DNA to be transcribed (52). Wt p53 exercises this activity in the context of components of the ATP-dependent nucleosomal remodeler SWI/SNF complex (53, 54). Mutant p53 has now also been identified to engage the SWI/SNF complex. However, in contrast to wt p53, mutant p53 is unable to directly engage wt p53 DNA response elements but rather localizes to distinct gene promoters through alternative transcription factors (as mentioned above). Through this co-recruitment, the SWI/SNF complex is predicted to facilitate more than 40% of all the genes transcribed by mutant p53 [where the primary example of altered regulation is the vascular endothelial growth factor receptor 2; VEGF2, which is vital for neoangiogenesis associated with oncogenesis (55)].

MLLs/MOZ

Mutant p53 can also alter transcriptional machinery, through distinct interactions from its wt counterpart. Wt p53 is able to physically interact through its core domain with the RNA polymerase II (POL II, large subunit) and limit target gene expression (56). In contrast, through engagement of the transcription factor ETS2 [as first demonstrated in Ref. (57)], mutant p53 is able to redirect POLII to transcribe the histone methyl transferases MLL1 and MLL2 and also acetyltransferase MOZ (58). This emphasizes the insidious capacity of mutant p53 to overpower fundamental transcriptional processes to support elevated proliferation. The newly emerging application of small molecule compounds to target chromatin regulators predicts application for cancers dependent on mutant p53. Specifically, cell growth inhibition of mutant p53 cancer has been demonstrated with prototoype inhibitors (58).

CONCLUSION

Corruption of the normal interactions between wt p53 and its molecular partners appears to lie at the heart of significant tumor promoting mutant p53 GOFs. Intriguingly, p53 mutations, which eliminate its function (e.g., deletion mutations), are rare, in contrast to the frequent activating missense mutations. To an extent, which appears unequaled by any other gene, mutation of p53 confers an exceptionally wide range of fundamental new properties that promote deregulated cell growth. These findings provide new insights directing innovative and rational approaches to therapeutically targeting cancers with mutant p53, which have proven particularly resistant to treatment. The polarized functions of these key p53 partners, would also caution that p53 status be an important criteria to consider prior to adoption of therapies directed toward these targets.

AUTHOR CONTRIBUTIONS

SH wrote the paper and prepared the figures. DR contributed to writing the paper and to preparing a figure. YH contributed to discussion and editing the paper.

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Mutant p53 and ETS2, a Tale of Reciprocity

*Luis Alfonso Martinez**

Department of Pathology, Stony Brook Cancer Center, Stony Brook University, Stony Brook, NY, USA

TP53 is one of the most frequently inactivated tumor suppressor genes in human cancer. However, unlike other tumor suppressor genes whose expression is lost, TP53 is usually inactivated as a result of a single nucleotide change within the coding region. Typically, these single nucleotide mutations result in a codon change that creates an amino acid substitution. Thus, unlike other tumor suppressor genes whose expression is lost due to genetic or epigenetic changes, the p53 gene primarily suffers missense mutations, and therefore, the cells retain and express a mutant form of the p53 protein (mtp53). It is now well established that mtp53 contributes to tumor development through its gain-of-function (GOF) activities. These GOF activities can arise from novel protein–protein interactions that can either disable other tumor suppressors (e.g., p63 and p73) or enable oncogenes such as ETS2, an ETS family member. In this review, I will focus on the identification of the mtp53/ETS2 complex and outline the diverse activities that this transcriptional regulatory complex controls to promote cancer.

Keywords: p53, Ets2, cancer, tumor suppressor protein p53, transcription factors

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***Correspondence:**
Luis Alfonso Martinez
Luis.Martinez@stonybrookmedicine.edu

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SIMULTANEOUS INACTIVATION OF WILD-TYPE p53's TUMOR SUPPRESSOR ACTIVITY AND ACTIVATION OF MUTANT p53's GOF ACTIVITIES

The majority of mutations in the p53 gene cluster within the region that encodes the DNA-binding domain. Some of these mutations alter the overall conformation of the protein (referred to as structural mutants), while other mutations do not alter the structure but instead change an amino acid that is critical for DNA binding (referred to as DNA contact mutants) (1, 2). These mutations typically give rise to mtp53 proteins that have lost the capacity to bind to the wild-type p53 (WTp53) consensus binding site and are thus unable to associate with WTp53 response elements in the genome and therefore unable to regulate WTp53 target genes. However, mtp53 is present on the promoters of various genes and is able to regulate their expression (1, 2). These observations indicate that despite having lost its WTp53 sequence-specific DNA-binding activity, mtp53 is still capable of acting like a transcription factor.

Initial studies on mtp53 relied on the overexpression of its cDNA in cells that were p53 null (3). In these studies, it was shown that mtp53 functions in a manner that is diametrically opposed to the tumor suppressor functions of WTp53. Instead of suppressing cancer or simply acting like an inert protein (due to its mutational inactivation), the mtp53 protein exhibited GOF activities, which allowed it to promote growth and tumorigenesis (3). From these studies, it became apparent that mtp53 can function as an oncogene, and these GOF activities were most apparent when the mtp53 harboring cells were challenged, for example, with proapoptotic stimuli (1, 2). The advent of siRNA technology permitted endogenous mtp53 to be suppressed in cells, which resulted in apoptosis (4). These data argued that mtp53 is actively engaged in promoting cell survival, and thus, cells harboring these

mutant proteins exhibit an addiction to them. Addiction to mtp53 has since been demonstrated by various groups and has recently been genetically confirmed in mice (5, 6). It is important to note from these latter studies *in vivo* that early intervention delays the tumorigenesis process, which could indicate that mtp53 is required to drive the carcinogenic pathway early on and possibly that addiction to this oncogene is an early occurring event, even in cells that are not yet "transformed." This latter view is supported by the fact that siRNA knockdown of mtp53 in non-transformed/non-tumorigenic Li–Fraumeni fibroblasts resulted in apoptosis, indicating that these cells exhibited oncogene addiction (4).

MECHANISTIC BASIS FOR MUTANT p53's TRANSCRIPTIONAL REGULATORY ONCOGENIC FUNCTIONS

To investigate the basis of its GOF activities, we and others have performed genome-wide analysis of mtp53 binding, and through bioinformatics and biochemical analysis have determined that mtp53 can be recruited to promoters *via* interactions with other transcription factors (7–11). Many of these transcription factors that bind to mtp53 have also been shown to interact with WTp53 (E2F1, NF-Y, VDR, ETS1, ETS2, and SP1), although there are some discrepancies among different studies (7–12). For example, one of the earliest studies to show that mtp53 regulates gene expression *via* the recruitment mechanism was on the regulation of the MDR1 promoter (13). In this study, it was reported that ETS1 can only interact with mtp53 and not with WTp53 (13). Other studies had shown that WTp53 can also interact with ETS1 (14, 15). ETS1 has been shown to be required for the transcriptional regulatory activity of WTp53 (16). Thus, it appears that both the oncogenic and tumor suppressor forms of p53 might rely on the ETS factors. We and others have reported that WTp53 at best poorly interacts with ETS1 (10, 11). What is the basis for the discrepancies between studies? It is possible that there might be tissue-specific or stress-dependent conditions that permit WTp53 to interact with ETS family members, although some studies were conducted *in vitro*. Additionally, WTp53 has been shown to undergo conformational changes during cell cycle progression. In this case, the protein can adapt mtp53-like conformational attributes (17–20). Does this reflect a conformational speciation of p53, where depending on cellular growth conditions or stress, p53 can adapt to different conformations that transiently increase its binding partners' repertoire. If this is the case, perhaps WTp53 can oscillate between different conformational species, a "minor" form of which is able to interact with ETS1. In contrast, mtp53 is locked in the minor form conformation that allows it to bind extensively to other protein partners including ETS1.

THE E26 TRANSFORMATION-SPECIFIC MOTIF IS OVERREPRESENTED IN MUTANT p53 OCCUPIED PROMOTERS

Chip-Chip and Chip-Seq data revealed that approximately 50% of the promoters occupied by mtp53 contain ETS-binding sites, suggesting that the association with ETS proteins is a prominent mechanism by which mtp53 regulates gene expression (10, 11). Mtp53 has been shown to associate with promoter regions of genes in an ETS2-dependent manner (10, 11). Importantly, these mtp53-bound genomic regions do not have a WTp53 response element, indicating that the mtp53 protein is not associating with these targets through residual activity of its DNA-binding domain (10, 11). Additionally, these mtp53 target genes do not overlap with WTp53 target genes that are identified through a similar genome-wide analysis, further demonstrating that mtp53 associates with these promoters in a completely novel manner (10, 11). Although the interaction with ETS1 might be important for the regulation of some mtp53 target genes, side-by-side comparison using recombinant proteins revealed that mtp53 preferentially associates with ETS2, another ETS family member (10). Both the structural and DNA contact p53 mutants interacted with ETS1, albeit with seemingly less affinity (10). It was also noted that the structural mutant (R175H) bound ETS1 better than the DNA contact (R248W) mutant. Importantly, all structural and DNA contact p53 mutants that have been tested thus far interact with ETS2 (10, 11). Moreover, whereas ETS1 knockdown generally has no effect on mtp53 target gene expression, ETS2 knockdown recapitulates the changes in gene expression that occur upon mtp53 knockdown (10, 11, 21, 22). Nevertheless, the observation that ETS2 interacts with various mtp53 (R175H, R248Q, R248W, R249S, R273H, R273L, and R280K) suggests that by coupling with ETS2, different mtp53 proteins are able to exert oncogenic activities through a common platform. The mtp53 proteins that have been tested correspond to the "hot-spot" mutations. It will be of interest to determine if proteins generated by missense mutations that are outside the region encompassed by the cluster of hot-spot mutations also interact with ETS2. However, further analysis is required to determine if all cancer-associated p53 mutants interact with ETS2. This is an important analysis because it has long been established that there are differences in the oncogenic potency of distinct p53 mutants, which might be related to their affinity for ETS2 or even other ETS factors. In this regard, it will be important to determine if p53 mutants that are more active than WTp53 in transcriptional and cell killing assays also interact with ETS2 (23).

It is of particular interest to note that ETS2 binds to the tetramerization domain of p53, which is thought to be functionally intact in both WTp53 and mtp53 (10). The question of how ETS2 distinguishes between mtp53 and WTp53 is further highlighted by the fact that some p53 mutants are considered to have subtle changes in their structure but are otherwise conformationally similar to WTp53. Intriguingly, it has been suggested that because WTp53 is actively engaged in sampling DNA sequences throughout the genome, it might not be able to interact with ETS2 (24). A corollary of this model would be that when WTp53 is associated with DNA, it might alter its structure in a manner that is incompatible with binding to ETS2. However, both overexpression and *in vitro* studies using recombinant proteins failed to show a strong interaction between WTp53 and ETS2. In the overexpression experiments, it seems unlikely that all of the transfected WTp53 protein is bound to DNA and thus cannot bind ETS2. Furthermore, the observation that the WTp53

and ETS2 purified proteins do not interact *in vitro* casts doubt on, yet does not eliminate, the possibility that the structural changes due to DNA binding by WTp53 prevent its interaction with ETS2 (10).

DOMAIN REQUIREMENTS FOR MUTANT p53's TRANSCRIPTIONALLY DEPENDENT GOF

Since WTp53 has potent transactivation domains in its N-terminus, this raises the possibility that mtp53 can also utilize them to regulate gene expression. In support of this, mutation of the N-terminal transactivation domain of mtp53 eliminated its ability to activate the MDR-1 promoter and enhances tumorigenic potential (25). A similar conclusion was drawn in another study, in which the N-terminus was shown to be required for the transactivation activity of mtp53 (26). In contrast, it was observed that the C-terminus was required for mtp53 to promote tumorigenicity (26). Likewise, an intact transactivation domain appears to be required for mtp53 to promote chemotherapy resistance (27, 28). It appears that mtp53 may be able to mediate GOF activities using different domains. The precise mechanism(s) by which these mutations disable its oncogenic activity is not well understood; however, it has been reported that mutation of the transactivation domain in mtp53 disrupts its interaction with ETS1 (13).

An mtp53, in which the transactivation domain was mutated, was still capable of activating the promoter of one of its target genes, TDP2, in a luciferase assay (10). An mtp53 mutant lacking the C-terminus, which eliminates the interaction with ETS2, was unable to activate this promoter (10). However, p53 contains two transactivation domains, and mutation of both domains prevents mtp53 from disrupting mammary tissue architecture *in vitro* (29). These observations suggest that both transactivation domains may be required for mtp53 to exert its GOF. However, in the latter case, mtp53 was mediating its effects through an interaction with SREBP transcription factor, which raises the possibility that the domains required for GOF are dependent on the particular binding partner for mtp53.

MUTANT p53 TAKES CARE OF ITS PARTNER

If the transcriptional activation domain of mtp53 is not required for activation of gene expression, what is mtp53 contributing to this transcriptional regulatory complex? Importantly, mtp53 can protect ETS2 from ubiquitin-dependent degradation, which raises the possibility that by increasing ETS2 abundance, mtp53 disrupts the balance between activator/repressor ETS family members, favoring the presence of mtp53/ETS2 on promoter targets (**Figures 1** and **2**) (10, 30). Among the different mtp53 interacting proteins, ETS2 appears to be unique in that mtp53 protects it from degradation. There are various different proteins currently implicated in promoting ETS2 degradation including Cdh1/Fzr1, the adaptor protein for the APC/cyclosome complex; Cul4a, a subunit of the SCF ubiquitin ligase complex; the E3-ubiquitin ligase, COP1/RFWD2; and CDK10 (31–34). Further work is required to establish how mtp53 interferes with the function of one or all of these proteins to stabilize ETS2.

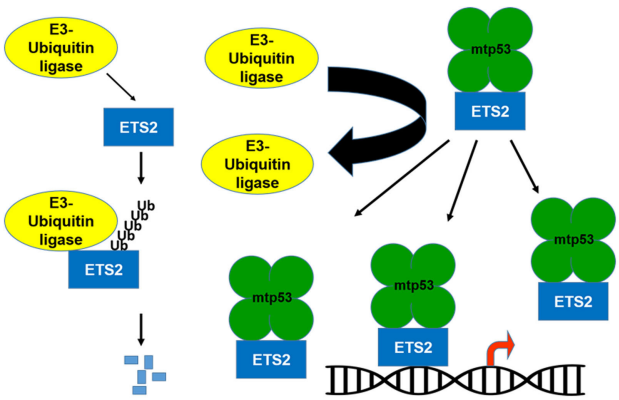

FIGURE 1 | Mutant p53 protects ETS2 from degradation. ETS2 is a labile protein with a short half-life. An E3-ubiquitin ligase binds to ETS2 and promotes its ubiquitin-dependent degradation. In the presence of mtp53, ETS2 is not ubiquitinated and becomes stable, which increases its abundance allowing it to recruit mtp53 to ETS target genes.

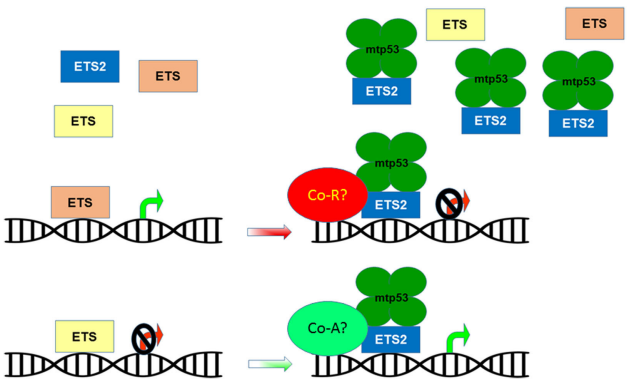

FIGURE 2 | Mutant p53 disrupts ETS family target gene regulation. The ETS family of transcription factors share multiple target genes based on their ability to bind a common DNA motif. These shared targets can be regulated in a cooperative or opposing manner in order to maintain homeostatic control of gene expression. The presence of mtp53 causes ETS2 to accumulate and outcompete other ETS family members for binding to target genes, potentially altering their repression/activation by recruiting co-repressors (Co-R) or co-activators (Co-A).

MUTANT p53's PARTNERING WITH ETS2 CONFERS IT ACCESS TO A MULTITUDE OF ONCOGENIC AND TUMOR SUPPRESSIVE TRANSCRIPTIONAL TARGETS

The ETS family of winged helix-turn-helix transcription factors consists of 28 family members that share a highly conserved

DNA-binding domain, referred to as the ETS domain (30). The ETS domain permits all the family members to bind in a sequence-specific manner to a common core motif, GGAA [called the ETS-binding site (EBS)], and thus, they share many transcriptional targets (30). This overlapping set of targets raises the question of why there is such redundancy in gene regulation. The perplexing redundancy in gene targets is explained by the fact that ETS family members largely exhibit tissue-specific expression and that they can play both cooperative and/or opposing roles in regulating gene expression (30, 35). As such, only a subset of ETS family members are expressed in a given tissue, and the particular ETS family member that is occupying a particular binding site is dependent on extracellular cues (36). Ectopic expression of oncogenic ETS proteins can functionally substitute for activation of the Ras/MAPK pathway, implying that control of oncogenic ETS factor levels is imperative to prevent neoplastic transformation (37). The ETS family regulates diverse cellular activities including apoptosis, angiogenesis, cell growth, adhesion, migration/invasion, the extracellular matrix, and other transcription factors (30). Thus, by interacting with ETS2, mtp53 can hijack the ETS transcriptional repertoire and control many of these processes to promote cancer.

As can be surmised from the various cellular activities that the ETS family controls, the ETS family members can function as either oncogenes or tumor suppressor genes, and sometimes the context determines their role in promoting or suppressing cancer. For example, ELF3 is frequently mutated in cervical, mucinous ovarian, and biliary tract cancers (38–40). Ectopic expression of wild-type ELF3 suppresses cell growth of biliary tract and cervical cancer cells suggesting a tumor suppressor role in these cancers (38, 39). In contrast, ELF3 has been shown to function as an oncogene in colorectal and prostate cancer (41, 42). SPDEF, a prostate epithelium-specific ETS transcription factor, suppresses prostate cancer progression and metastasis (43–45). Knockdown of another ETS family member, ESE3/EHF, in normal prostate cells resulted in the acquisition of mesenchymal and stem-like characteristics (46). Chromosomal rearrangements have been shown to give rise to oncogenic gene fusions for multiple ETS family members including ERG, ETV1, ETV4, ETV5, ETV6, ELK4, and FLI1 (47). Importantly, ETS2 itself has been shown to be deleted in a subset of prostate cancers and to have a growth inhibitory function, suggesting that it is a tumor suppressor gene in this tissue. In addition, a transgenic mouse overexpressing ETS2 in the thymus had increased p53-dependent apoptosis (48). Previously, it has been shown that Ets2 dosage can impact tumor development in the APC^{Min} mouse model (49). Mice carrying extra copies of ETS2 were protected from tumor development, whereas ETS2 heterozygous mice exhibited higher cancer frequency (49). It is interesting to note that in the context of mutant p53 harboring cells, ETS2 abundance is increased yet it appears to function as an oncogene. Given that ETS2 has been shown to activate p53-dependent apoptosis, it is possible that the loss of wild-type p53 provides a permissive environment for ETS2 to have oncogenic functions. Taken together, the ability of mutant p53 to stabilize ETS2 and to utilize it to regulate gene expression constitutes a novel mechanism by which an ETS family member promotes cancer.

ALTERED TARGET SELECTION VS. AMPLIFIED REGULATION

There are various aspects of the mtp53/ETS2 regulatory complex that remain to be explored. For example, are the genes regulated by mtp53/ETS2 different from the ones regulated by ETS2 alone? Additionally, does the mtp53/ETS2 interaction alter the regulation (i.e., activation or repression) of these target genes? Since ETS2 is induced by growth factor receptor pathways, does mtp53 unlink it from mitogenic signaling and thereby produce a constitutively active ETS2. A clue comes from the observation that many of the mtp53/ETS2 target genes are controlled by ETS2 in cells lacking p53 (21, 22). This suggests that mtp53 is not altering the spectrum of genes that ETS2 controls but rather further enhancing their expression. For example, mtp53 was shown to upregulate nucleotide metabolism genes (NMG) expression by associating with their promoters, and suppression of mtp53 or ETS2 reduced their expression (22). In cells lacking mtp53 (i.e., either containing WTp53 or lacking p53 altogether), ETS2 knockdown reduced the expression of these target genes (22). Introduction of mtp53 increased ETS2 protein and NMG expression to levels higher than in cells lacking mtp53 (22). These data reinforce the notion that the mtp53/ETS2 complex upregulates NMG expression. Again, in this situation, ETS2 knockdown in cells ectopically expressing mtp53 reduced NMG expression, despite the fact that it did not affect mtp53 levels (22). These data suggest that the mtp53-mediated aberrant accumulation of ETS2 can enhance the expression of ETS2 target genes. In addition, removal of mitogens (*via* serum deprivation) results in reduced expression of the NMG in cells lacking mtp53, yet has no effect in cells expressing mtp53 (22). This observation raises the possibility that mtp53 is capable of superseding the mitogenic control of ETS2 function. Whether mtp53 is obviating intrinsic ETS2 auto-inhibitory activity or simply increasing its abundance, or both, to enhance ETS2 function requires further investigation (50).

FUTURE DIRECTIONS

The cooperation between mtp53 and ETS2 to regulate gene expression is well established *in vitro*, but the extent to which these two work together to promote tumorigenesis *in vivo* is still not known. Furthermore, there is circumstantial evidence that mtp53's GOF depends on several domains, and thus, it will be important to dissect these different domains *in vivo* to determine if one of these is dominant or whether the GOF is mediated by the action of multiple domains in mtp53.

AUTHOR CONTRIBUTIONS

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MINI REVIEW
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Che-1/AATF: A Critical Cofactor for Both Wild-Type- and Mutant-p53 Proteins

*Tiziana Bruno, Simona Iezzi and Maurizio Fanciulli**

SAFU Laboratory, Department of Research, Advanced Diagnostic, and Technological Innovation, Regina Elena Cancer Institute, Rome, Italy

The p53 protein is a key player in a wide range of protein networks that allow the state of "good health" of the cell. Not surprisingly, mutations of the TP53 gene are one of the most common alterations associated to cancer cells. Mutated forms of p53 (mtp53) not only lose the ability to protect the integrity of the genetic heritage of the cell but also acquire pro-oncogenic functions, behaving like dangerous accelerators of transformation and tumor progression. In recent years, many studies focused on investigating possible strategies aiming to counteract this mutant p53 "gain of function" but the results have not always been satisfactory. Che-1/AATF is a nuclear protein that binds to RNA polymerase II and plays a role in multiple fundamental processes, including control of transcription, cell cycle regulation, DNA damage response, and apoptosis. Several studies showed Che-1/AATF as an important endogenous regulator of p53 expression and activity in a variety of biological processes. Notably, this same regulation was more recently observed also on mtp53. The depletion of Che-1/AATF strongly reduces the expression of mutant p53 in several tumors *in vitro* and *in vivo*, making the cells an easier target for chemotherapy treatments. In this mini review, we report an overview of Che-1/AATF functions and discuss a possible role of Che-1/AATF in cancer therapy, with particular regard to its action on p53/mtp53.

Keywords: Che-1/AATF, p53, apoptosis, survival

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Ygal Haupt,
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Afshin Samali,
National University of Ireland, Ireland
Takaomi Sanda,
National University of Singapore, Singapore

***Correspondence:**
Maurizio Fanciulli
fanciulli@ifo.it

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The *TP53* gene is a tumor suppressor capable of detecting oncogenic events in tumor cells and eliminating them through using several different mechanisms. It is the most frequently mutated gene in human cancers, and p53 mutant forms (mtp53), in addition to losing the function of the wild-type p53 as "guardian of the genome," acquire specific properties that contribute to the aggressiveness and chemoresistance of cancer (1). The activity of wild-type p53 is modulated through various mechanisms, which contribute to its full functionality, regulating both its stability and its specificity of action. Notably, these same mechanisms also operate on mtp53, sustaining its oncogenic functions (2–4). Che-1/AATF was recently identified among the proteins that can not only regulate p53 functions but also support the activity of oncogenic mtp53. In this mini review, we provide an updated overview of Che-1/AATF activities, detailing its intimate connection with p53.

Che-1/AATF

Che-1/AATF was identified in the early years of this decade by different groups both as a protein involved in the control of transcription and apoptosis, and a gene downregulated upon TGFβ

induced differentiation (5–8). This protein is able to connect specific transcription factors to the general transcriptional machinery through its interaction with the subunit 11 of RNA polymerase II (hRPB11) (6). In particular, Che-1/AATF has been shown to interact and modulate the activity of several nuclear hormone receptors (9) and transcription factors, including the retinoblastoma protein (pRb), p65 and STAT3 (10–12). These interactions are mostly regulated by post-translational modifications, which provide a rapid and reversible manner to modulate Che-1/AATF co-transcriptional activity in response to different stimuli. Moreover, Che-1/AATF action on transcription may also be modulated by its binding to different forms of hRPB11. Indeed, this subunit is the product of a multigene family, which encodes specific proteins differently expressed in several tissues and showing different binding capacities (13, 14). Che-1/AATF protein is found expressed in all tissues (6, 7), and its expression is required for proliferation and survival. Indeed, Traube (Che-1/AATF mouse orthologous) knock out mice halt the development at the compacted morula stage and are embryonically lethal. Furthermore, mutant embryos exhibit a reduction in cellular proliferation (15), indicating Che-1/AATF's involvement in cell cycle regulation. Consistent with these observations, Che-1/AATF has been shown to be involved in cell cycle progression through its ability to affect pRb protein's growth suppression functions (10, 16). Moreover, it was demonstrated that Che-1/AATF localizes at interphase centrosomes and regulates centrosome duplication and spindle formation indicating a role for Che-1/AATF in the control of mitotic entry (17). Che-1/AATF not only regulates cellular proliferation but also has a significant role in controlling the apoptotic process. To date, most of the information regarding the antiapoptotic function of Che-1/AATF derives from studies performed in the neural tissue, where this protein appears to take part in regulating apoptotic activation in both physiological and pathological conditions (18–21). Moreover, Che-1/AATF interacts with cytoplasmic Tau in rat cerebellar granule neurons, and this interaction is modulated during neuronal apoptosis (22). A protective role of Che-1/AATF has also been described in human kidney proximal tubule cells, where this protein antagonizes apoptotic cell death by preserving mitochondrial function and reducing oxidative damage (23). Alternatively, Che-1/AATF has also been reported to have a pro-apoptotic role. Indeed, Che-1/AATF overexpression increases UV-induced apoptosis by promoting phosphorylation and transcriptional activity of the apoptotic gene c-Jun, in a p53 independent way. Moreover, UV damage induces Che-1/AATF redistribution from the nucleolus to the nucleoplasm, thus allowing Che-1/AATF and c-Jun to directly interact (24).

Che-1/AATF WITHIN WT-p53 TUMOR SUPPRESSOR ACTIVITIES

The tumor suppressor p53 is one of the main effector of the DNA damage response (DDR), a complex network of pathways responsible for maintaining genome integrity and preventing tumorigenesis (25, 26). DDR coordinates several pathways that cooperate together to detect DNA lesions, arrest cell cycle in order to allow repair, and induce apoptosis or senescence if damage is too severe (27). p53 is a key signal integrator of these pathways, capable of regulating the transcription of a large variety of target genes, and for this reason, its levels and activities are tightly regulated inside the cell. Upon DNA damage, p53 expression can be enhanced at both transcriptional (28) and translational level (29). However, its functions are largely modulated by post-translational modifications and protein–protein interactions (30). In the last past years, several studies have identified Che-1/AATF as an important component of DDR and an endogenous p53 regulator (8). In response to genotoxic stress, Che-1/AATF is extensively modified by post-translational modifications affecting its localization, half-life, and interactions (8). Among these modifications, phosphorylation by checkpoint kinases ATM and Chk2 has a pivotal role in the context of Che-1/AATF-p53 connection. Indeed, this modification greatly affects Che-1/AATF functions, acting as a molecular switch that moves this protein from the pathways promoting cell cycle progression to the ones involved in cell cycle arrest and survival. In particular, (ATM–Chk2) phosphorylated-Che-1/AATF interacts with NF-kB p65 subunit, and this interaction moves Che-1/AATF from E2F1-dependent promoters to the *TP53* promoter, thus increasing transcription of this gene and contributing to the increase of p53 protein levels after genotoxic stress (11). Notably, phosphorylated-Che-1/AATF activates p53 transcription also in the absence of genotoxic stress, probably as a consequence of an intrinsic DNA damage occurring during DNA replication (11). This observation leads to hypothesize a model in which Che-1/AATF is already required for the basal state of p53 expression, and this activity is reinforced in response to DNA damage. Moreover, Che-1/AATF plays an important role in the maintenance of the G2/M checkpoint, and this effect depends on the activation of p53. Consistent with these findings, Che-1/AATF depletion was found to sensitize cancer but not normal cells to antineoplastic drugs (11).

In addition to sustaining *TP53* transcription, Che-1/AATF phosphorylation also promotes the binding of Che-1/AATF to p53, regulating in such way p53 activities (31). Of interest, Che-1/AATF is a component of a ternary complex with p53 and Brca1, and p53 is required for these interactions. This complex is observed at the early stage of the DDR, and when DNA damage is too extensive and cells undergo apoptosis, p53 modifications produced by Pin1 induce the detachment of the proteins. Notably, the interaction between Che-1/AATF-p53 specifically directs p53 toward the transcription of genes involved in growth arrest over its pro-apoptotic target genes. Indeed, a Chip-Seq analysis revealed a strong enrichment of p53 target genes involved in apoptosis in Che-1/AATF depleted cells, with a concomitant decrease in genes regulating growth arrest (31).

Höpker et al. have described another mechanism by which Che-1/AATF modulates p53 activity. They highlighted a cytoplasmic localization of Che-1/AATF in absence of DNA damage, and demonstrate that in response to genotoxic stress, this protein translocates from the cytoplasm to the nucleus, as a consequence of a phosphorylation by the checkpoint kinase MK2 (32). Remarkably, nuclear Che-1/AATF regulates the cellular outcome of the p53 response by competing with this protein for the binding to the promoter of several apoptotic genes, inhibiting in such way their activation (32).

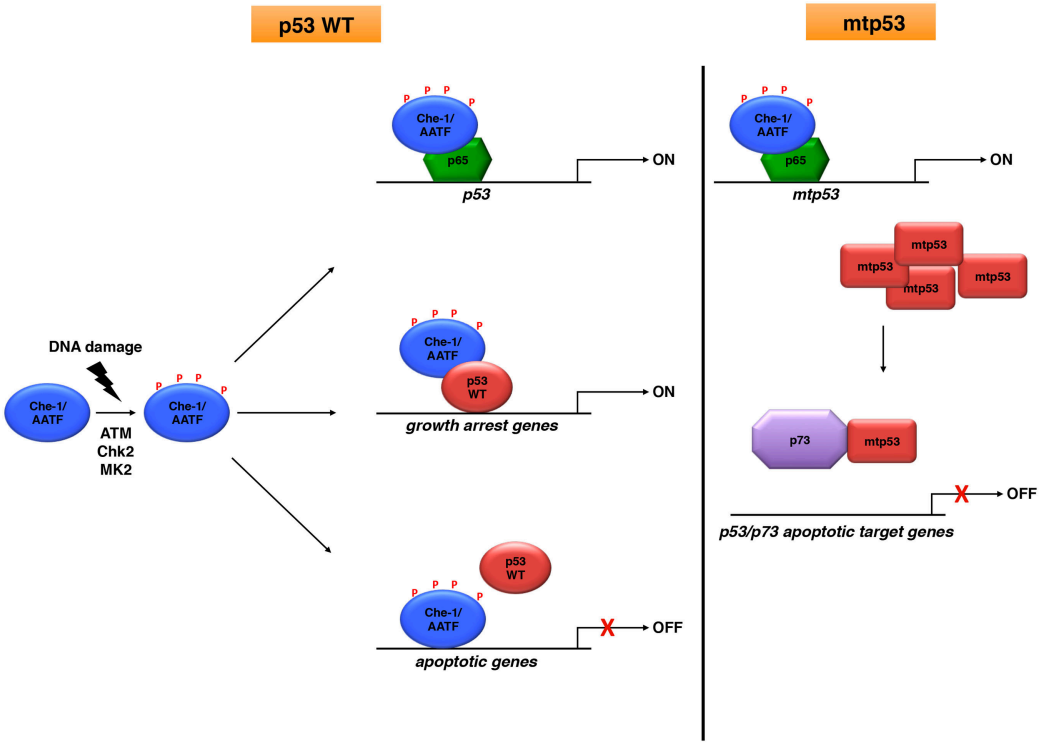

FIGURE 1 | Che-1/AATF is an endogenous regulator of p53 activities. Che-1/AATF promotes cell survival in response to DNA damage by regulating both transcription and activity of p53. However, in tumor cells carrying p53 mutations, Che-1/AATF sustains mtp53 levels and promotes p73/mtp53 complex formation, inhibiting in such way the apoptotic activity of p73.

Consistent with all these findings, Che-1/AATF$^{+/-}$ mice exhibited a greater apoptosis in response to genotoxic stress when compared to wild-type littermates. Furthermore, thymocytes from Che-1/AATF$^{+/-}$ mice showed an increase of p53 protein on pro-apoptotic gene promoters (31), thus confirming that Che-1/AATF controls p53 activity both *in vitro* and *in vivo*.

A further indication of the intimate relationship between Che-1/AATF and p53 arises from the observation that p53 binds the promoter of *Che-1/AATF* gene in response to DNA damage, leaving to assume the existence of a regulatory feedback loop between the two proteins (31). Moreover, there have been numerous findings that showed how many pathways operating on p53 are actually involved in Che-1/AATF regulation. In fact, the pro-apoptotic kinase HIPK2 phosphorylates Che-1/AATF at residue T144 in response to apoptotic DNA damage. This modification permits the prolyl isomerase Pin1 to produce a conformational change, facilitating the interaction with ubiquitin ligase HDM2, thereby inducing Che-1/AATF ubiquitylation and proteasomal degradation (33, 34). Notably, not only does Che-1/AATF activate the transcription of p53 and regulate its functions but also it is able to strengthen p53 functions through parallel pathways. For instance, p53 inhibits the kinase mTOR, in response to DNA damage through sestrin1 and 2 activation (35). A recent study has shown that Che-1/AATF inhibits mTOR activity in a p53 independent way, by increasing the transcription of the mTOR inhibitor Redd1 and Deptor in response to different types of cellular stress (36).

Che-1/AATF ENHANCES THE ONCOGENIC POTENTIAL OF MUTANT p53 PROTEINS

As previously described, Che-1/AATF regulates p53 functions in response to DNA damage by increasing its expression and regulating p53 promoter selection. However, very often the proteins involved in p53 activation are also able to sustain and amplify the "gain-of-function" of mtp53 in tumors containing mutated forms of this protein (1). In this regard, Che-1/AATF has shown to play an important role on the activity of the mutant forms of p53. In several breast carcinoma cell lines carrying different forms of mtp53, Che-1/AATF was found accumulated and recruited onto the *TP53* promoter, whereas it was almost undetectable in primary breast epithelial cells (37). According to these findings, Che-1/AATF is required in sustaining mtp53 expression, and its depletion strongly decreases mtp53 expression both at mRNA and protein level, inducing apoptosis without involving any other stimuli. In addition, depletion of Che-1/AATF significantly reduces the expression of important genes involved in DNA repair in cells expressing mtp53, such as BLM and Rad17, inducing in such way endogenous DNA damage and triggering p73

expression as well as its target apoptotic genes *Noxa* and *Puma* (37). It is important to note that, Che-1/AATF depletion did not activate apoptosis in normal cells or in tumor cells carrying either WT-p53 or lacking p53 expression, thereby suggesting that these phenomena require mtp53 downregulation.

It has been widely shown that a major oncogenic ability of the mtp53 proteins is their ability to activate an aberrant transcription of selected target genes involved in cell proliferation by interacting with several transcription factors and being recruited on regulatory regions of chromatin (38). Therefore, it is possible to assume that similar to wild-type p53, this interaction may contribute to aberrant gene regulation conducted by mtp53.

Che-1/AATF AS A PUTATIVE THERAPEUTIC TARGET IN CANCER

All the observations described above indicate that Che-1/AATF plays a prominent role in many aspects of cancer biology. Even though mutations of Che-1/AATF have not been described so far (39), several studies reported an increase of Che-1/AATF levels in some types in cancer. In particular, elevated levels of this protein have been found in several leukemia cell lines (40) and in patients with chronic lymphocytic leukemia (41) or multiple myeloma (36). In addition, Che-1/AATF gene was found amplified in neuroblastoma patients most of whom expressing wild-type p53, and high levels of Che-1/AATF were found correlated with poor prognosis and reduced survival (32). Importantly, not only this protein is involved in cell cycle progression and in protecting cancer cells from apoptosis induction but also able to control p53 activity (11, 32), inhibiting p53 mediated transcription of apoptotic genes (31, 32). Moreover, Che-1/AATF strongly supports the "gain of function" of the mutated forms of this oncosuppressor (**Figure 1**) (37). Altogether, these observations indicate that dysregulation of Che-1/AATF expression level could be relevant for the transformation process, and strengthen the notion that Che-1/AATF could be considered a valid target for novel anticancer therapeutic approaches either in tumors expressing wild-type p53, or in cancers carrying its mutated forms. In agreement, Che-1/AATF depletion was shown to increase sensitivity to anticancer agents both *in vitro* and *in vivo* (11, 32, 42), and to activate the apoptotic process in cancer cells carrying mtp53 (37). Unfortunately, no compounds capable of inhibiting Che-1/AATF activity have been identified so far. However, future studies focusing on understanding the mechanisms of action of Che-1/AATF and the characterization of the pathways implicated in its regulation will provide useful indications toward developing specific inhibitors for this protein.

AUTHOR CONTRIBUTIONS

TB, SI, and MF equally contributed to write this mini review.

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REVIEW
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Targeting Oncogenic Mutant p53 for Cancer Therapy

*Alejandro Parrales and Tomoo Iwakuma**

Department of Cancer Biology, University of Kansas Medical Center, Kansas City, KS, USA

Among genetic alterations in human cancers, mutations in the tumor suppressor *p53* gene are the most common, occurring in over 50% of human cancers. The majority of p53 mutations are missense mutations and result in the accumulation of dysfunctional p53 protein in tumors. These mutants frequently have oncogenic gain-of-function activities and exacerbate malignant properties of cancer cells, such as metastasis and drug resistance. Increasing evidence reveals that stabilization of mutant p53 in tumors is crucial for its oncogenic activities, while depletion of mutant p53 attenuates malignant properties of cancer cells. Thus, mutant p53 is an attractive druggable target for cancer therapy. Different approaches have been taken to develop small-molecule compounds that specifically target mutant p53. These include compounds that restore wild-type conformation and transcriptional activity of mutant p53, induce depletion of mutant p53, inhibit downstream pathways of oncogenic mutant p53, and induce synthetic lethality to mutant p53. In this review article, we comprehensively discuss the current strategies targeting oncogenic mutant p53 in cancers, with special focus on compounds that restore wild-type p53 transcriptional activity of mutant p53 and those reducing mutant p53 levels.

Keywords: mutant p53, depletion, compounds, reactivation, cancer therapy, gain of function, dominant negative, oncogenes

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Peter MacCallum Cancer Centre, Australia

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***Correspondence:**
Tomoo Iwakuma
tiwakuma@kumc.edu

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INTRODUCTION

The tumor suppressor p53 exerts its biological function by regulating transcription of numerous downstream target genes involved in cell cycle arrest, apoptosis, DNA repair, senescence, and metabolism as a transcription factor (1, 2). p53 is also directly recruited to the mitochondria and induces apoptosis independent of its function as a transcription factor (3). Under unstressed physiological conditions, p53 expression is maintained at a low level, mainly by being degraded by its E3 ubiquitin ligases, MDM2, Pirh2, and COP1 (4). Once cells are exposed to genotoxic stresses, p53 is posttranslationally modified through phosphorylation and acetylation, becomes stabilized, and induces cell cycle arrest and/or cell death. When p53 activity is lost by gene deletion or mutations, normal cells lose the abilities to control their growth and death, leading to immortalization and ultimately cancer (5). The observation that over 50% of human cancers have mutations in the *p53* gene indicates the indispensability of intact p53 activity for suppressing tumor development (6).

Mutations in the *p53* gene occur mainly in the DNA-binding domain, the majority of which are missense mutations, resulting in loss of function as a transcription factor and accumulation of dysfunctional p53 protein in tumors (7). Mutant p53 can be categorized roughly into two types:

DNA contact (class I) mutant where mutations are present on amino acids directly binding to the p53-responsive element in DNA (e.g., $p53^{R273H}$ and $p53^{R280K}$) and conformational (class II) mutant in which mutations alter structure of p53 to abolish its DNA-binding ability (e.g., $p53^{R175H}$ and $p53^{V143A}$) (8). Both the mutant types not only lose the transcriptional activity, but also have the dominant-negative (DN) activity by hetero-oligomerizing with wild-type p53. Moreover, mutant p53 shows oncogenic gain-of-function (GOF) activities, such as enhanced tumor progression, metastatic potential, and drug resistance, when overexpressed even in cells lacking wild-type p53 (7). These findings are supported by the fact that p53 was originally appreciated as an oncogene, since researchers unknowingly used plasmids encoding mutations in the *p53* gene. Thus, mutant p53 functions as an oncogene and greatly contributes to malignant properties of cancer cells.

Disrupting specific mechanisms which cancer cells develop for their survival and growth is a rational approach to selectively kill cancer cells with minimal effects on normal cells. In this regard, mutant p53 is one of the best druggable targets, since over half of human cancers have p53 mutations, while normal cells mostly do not have mutations in the *p53* gene (9). To exploit the frequent presence of mutant p53 in tumors and target mutant p53 in cancer therapy, two strategies including restoration of wild-type p53 transcriptional activity and depletion of mutant p53 have been extensively undertaken, in addition to inhibition of downstream target pathways involved in mutant p53 GOF and induction of synthetic lethality to mutant p53. Since mutant p53 is generally accumulated in tumors (10), reactivating p53 activity can efficiently induce proliferation arrest and/or cell death of cancer cell. Specifically, in the late stage of tumor development, cancer cells express only mutant p53 with loss of heterozygosity of the other wild-type *p53* allele (11, 12). Such cells often have high metastatic and chemotherapy resistant properties. Hence, this p53 reactivation strategy is powerful to treat cancers expressing mutant p53. The other strategy to specifically deplete oncogenic mutant p53 in cancer cells should have minimal impact on wild-type p53, since depletion of wild-type p53 in normal and cancer cells can accelerate tumorigenesis or tumor progression. Accumulating studies suggest that knockdown of mutant p53 significantly reduces oncogenic potential of cancer cells expressing only mutant p53 (13–16), suggesting that malignant properties of cancer cells are, at least partially, dependent on the presence of mutant p53. This could be simply due to the loss of oncogenic activity of mutant p53 or possibly because cancer cells are addicted to mutant p53 for their survival and proliferation. This strategy would work even better when cancer cells retain the wild-type *p53* allele with the mutant *p53* allele (heterozygous), since it can also restore wild-type p53 activity which is suppressed by the DN activity of mutant p53. Thus, depletion of mutant p53 is also an effective strategy to suppress tumor progression.

In this article, toward developing precision cancer medicine, we summarize updated information about compounds which can restore wild-type p53 activity, as well as those depleting mutant p53.

DRUGS/COMPOUNDS THAT RESTORE WILD-TYPE p53 ACTIVITY

Most p53 mutants lose their ability to bind with p53-response elements in DNA, thereby losing transcriptional activity and tumor suppressive function (17). However, the following evidence suggests that sequence-specific p53 transcriptional activities can be restored from mutant p53: (1) many p53 mutants are temperature sensitive and restore the p53 activity at the permissive temperature (18, 19), (2) synthetic peptides, CDB3 and Peptide 46 which are derived from 53BP2 and C-terminal domain of p53, respectively, restore the sequence-specific DNA binding and transcriptional activity of p53 (20, 21), and (3) insertion of second-site mutations or a N-terminal deletion in several p53 mutants restore the p53 transcriptional activities (22–24). Since the first p53-reactivating compound, CP-31398, was identified (17), investigators have made tremendous efforts to identify compounds that restore p53 transcriptional activity. Major compounds related to mutant p53 reactivation are listed in **Table 1** and explained below.

CP-31398

CP-31398 (styrylquinazoline) was identified through a structure-based screening as a compound which could restore native wild-type p53 conformation from a denatured conformation in the DNA-binding domain, using a conformation specific antibody PAb1620. CP-31398 leads to increase in *p21* mRNA expression in Saos-2 (p53-null) cells expressing $p53^{V173A}$ and $p53^{R249S}$ mutants, and inhibits tumor growth of A375.S2 ($p53^{R249S}$) and DLD1 ($p53^{S241F}$) cells (17). CP-31398 increases mRNA expression of *MDM2* and *p21* in multiple cancer cell lines (46). CP-31398 also induces mitochondrial translocation of mutant $p53^{R273H}$ in A431 skin carcinoma cell line, leading to cytochrome c release and apoptosis (25). Intriguingly, CP-31398 cannot refold already misfolded mutant p53 proteins, since cycloheximide prevents the effect of CP-31398 on p53 restoration (23, 26). It also induces cell death in a p53-independent manner through free radical formation (27).

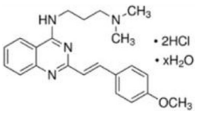
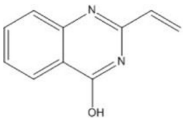
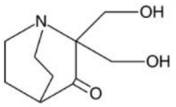
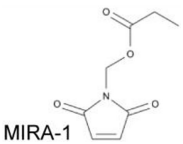
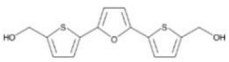
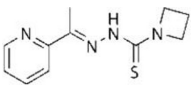
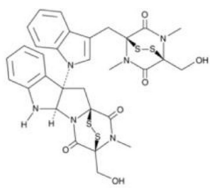
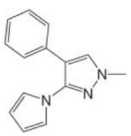
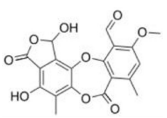
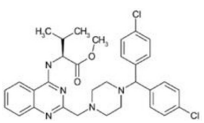
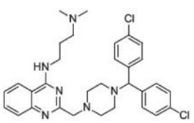
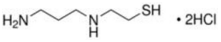
STIMA-1 (SH Group-Targeting Compound That Induces Massive Apoptosis)

STIMA-1 [2-vinylquinazolin-4-(3H)-one] was identified as one of the CP-31398 derivatives, which induced mutant p53 ($p53^{R175H}$ and $p53^{R273H}$)-dependent growth suppression (28). Both CP-31398 and STIMA-1 bind to the cysteine residues in the core domain of mutant p53, leading to stabilization of wild-type p53 conformation and subsequent restoration of transcriptional activity (28). STIMA-1 increases the DNA-binding ability of mutant p53, resulting in upregulation mRNA expression of *p21*, *PUMA*, and *BAX*, and leading to mutant p53-dependent apoptosis (28).

PRIMA-1 and PRIMA-1^{MET}/APR-246

PRIMA-1 [2,2-bis (hydroxymethyl)-3-quinuclidinone] was identified through a screening as a compound that suppressed proliferation of Saos-2 osteosarcoma cell line expressing $p53^{R273H}$ (Saos-2-$p53^{R273H}$) with little effect on the parental Saos-2 cells.

TABLE 1 | Compounds that induce reactivation of mutant p53.

Compound	Type of mutant	Mechanism	Reference	Structure
CP-31398	V173A, S241F, R249S, R273H	Stabilize the DNA-binding core domain and induce conformational change	(17, 25–27)	
STIMA-1, structural similarity to CP-31398	R175H, R273H	Bind to the cysteine residues in the core domain and stabilize wild-type p53 conformation	(28)	
PRIMA-1 and the methylated analog (APR-246/PRIMA-1^{MET})	R175H, R273H	Bind to thiol groups in the core domain and restore wild-type conformation	(23, 29, 30)	 PRIMA-1
MIRA-1 (NSC19630), and its analogs MIRA-2 and -3	R175H, R248Q, R273H	Prevent unfolding of wild-type and mutant p53 and restore native wild-type p53 conformation	(31)	 MIRA-1
RITA (NSC652287)	R175H, R248W, R273H, R280K	Restore p53 transcriptional activity and induce apoptosis	(32, 33)	
NSC319726/ ZMC 1 (zinc metallochaperone-1)	R175H, R172H (mouse)	Restore wild-type p53 conformation and activity with MDM2-dependent degradation	(34–36)	
Chetomin (CTM)	R175H	Increase Hsp40 (DNAJB1) levels and Hsp40-p53^{R175H} binding, restoring wild-type p53 conformation, activity, and MDM2-dependent degradation	(37)	
PK7088	Y220C	Bind to a p53^{Y220C}-specific surface cavity and stabilize p53^{Y220C} with restored wild-type p53 conformation	(38)	
Stictic acid (NSC87511)	R175H, G245S	Target cysteine 124 at the p53 core domain and restore wild-type p53 activity	(39)	
p53R3	R175H, M237I, R273H	Restore sequence-specific DNA binding and p53 transcriptional activities	(40)	
SCH529074	R175H, L194F, R248W, R249S, R273H	Restore sequence-specific DNA binding and p53 transcriptional activities	(41)	
WR-1065	V272M	Restore DNA binding and transcriptional activities of p53^{V272M}	(42–45)	

PRIMA-1 and its methylated analog PRIMA-1^{MET} (also known as APR-246) not only enhance stability of wild-type p53 at 37°C, but also induce conformational change of p53^{R175H}, leading to restoration of DNA-binding activity of p53^{R175H} with increased *MDM2* and *p21* mRNA expression (23). Notably, PRIMA-1 refolds previously accumulated unfolded mutant p53 (23). The

mechanisms underlying refolding of mutant p53 by PRIMA-1 and PRIMA-1^{MET} involve the conversion of these compounds to products which form adducts with thiol groups in the mutant p53 core domain, leading to restoration of wild-type conformation and induction of apoptosis in tumor cells (29, 30). Several studies have successfully validated their tumor suppressive effects in mouse models of multiple types of cancer (47–50). Importantly, PRIMA-1^{MET} is currently in clinical trials (51, 52).

MIRA-1 and Its Structural Analogs

Using the same screening strategy as PRIMA-1, MIRA-1 (NSC19630) was identified as a compound that suppressed proliferation of Saos-2-p53^{R273H} cells (31). MIRA-1 and its structural analogs MIRA-2 and MIRA-3 from the NCI repository inhibit proliferation of cancer cells expressing p53^{R175H} and p53^{R273H} (31). Both MIRA-1 and MIRA-3 also induce cell death in cancer cells expressing mutant p53 (31). Furthermore, MIRA analogs prevent unfolding of wild-type and mutant p53, and also restored native wild-type p53 conformation, leading to enhanced DNA-binding activity of mutant p53 (p53^{R175H} and p53^{R248Q}) and increase in mRNA expression of p53 downstream target genes, *MDM2* and *p21*, in several mutant p53-carrying cancer cell lines (31). *In vivo* effects of MIRA analogs have also been confirmed in mouse models (31, 53).

RITA (NSC652287)

NSC652287 [2,5-bis(5-hydroxymethyl-2-thienyl) Furan] is one of a series of thiophene derivatives and is known to inhibit tumor growth of renal cell carcinoma cells with DNA–protein cross-linking and upregulation of wild-type p53 and p21 (54, 55). NSC652287 (RITA: reactivation of p53 and induction of tumor cell apoptosis) is also identified through cell proliferation assay-based screening using isogenic cell lines of HCT116 (wild-type p53 and p53-null) as a compound that suppresses the growth of HCT116 (wild-type p53) cells in a dose-dependent manner with minimum effects on HCT116 (p53-null) cells (56). Later, NSC652287/RITA was found to suppress the growth of cancer cell lines carrying various p53 mutants (p53^{R175H}, p53$^{R213Q/Y234H}$, p53^{R248W}, p53^{R248Q}, p53^{I254D}, p53^{R273H}, and p53^{R280K}) by restoration of p53 transcriptional activity (*p21*, *NOXA*, *PUMA*, and *GADD45*) and induction of apoptosis through upregulation of pro-apoptotic proteins and downregulation of several oncogenes or anti-apoptotic proteins (32, 33, 57). However, the exact mechanism by which RITA activates both wild-type and mutant p53 to induce apoptosis remains unclear.

NSC319726/ZMC1 (Zinc Metallochaperone-1)

Zinc is required for proper folding of p53 protein, while lack of zinc in the central core domain of p53 leads to unfolding (23, 58, 59). Also, addition of zinc to cells or its administration to mice carrying tumors are known to restore DNA-binding activity of mutant p53 (p53^{R175H} and p53^{R273H}) in cells and tumors, leading to tumor suppression (60). Thus, facilitating the binding of mutant p53 to zinc can be used to restore the proper folding and transcriptional activity of mutant p53 (34). Indeed, NSC319726 [zinc metallochaperone-1 (ZMC1)], a thiosemicarbazone derivative, was identified in a screen of the NCI60 panel of human tumor cell lines as a compound that exhibited selective toxicity to cells carrying p53^{R175H} with minimum effects on cells expressing wild-type p53 and other p53 mutants (p53^{R248Q} and p53^{R273H}) (35). NSC319726 restores the wild-type-like conformation of mutant p53 and upregulates p53 downstream target genes (*p21*, *PUMA*, and *MDM2*) through increasing ROS levels (35, 36). It also reduces p53^{R175H} levels, which is rescued by Nutlin-3a (35). Importantly, its administration induces greater toxicity in *p53$^{R172H/R172H}$* (equivalent to human p53^{R175H}) mice than in wild-type mice in a dose-dependent manner (35). NSC319726 also suppresses tumor growth of TOV112D (p53^{R175H}) cells *in vivo* (35). Other thiosemicarbazone family members (NSC319726 and NSC328784) also preferentially reduce cell viability of p53 mutant cell lines (35).

Chetomin

Hiraki et al. (37) performed high-throughput chemical library screening using a luciferase reporter with the p53 response element of the *PUMA* promoter in H1299 (p53-null) cells carrying p53^{R175H}, which identified chetomin (CTM) as a compound that increased luciferase activity dose-dependently. CTM suppresses cancer cell growth *in vitro* and *in vivo* more efficiently in cells expressing p53^{R175H} with upregulation of MDM2, p21, and PUMA than those expressing wild-type p53 and p53^{R273H}, as well as null for p53 (37). The effects of CTM on reduced p53^{R175H} levels are dependent on MDM2 (37). Interestingly, CTM increases protein levels of Hsp40 (DNAJB1) and the binding of p53^{R175H} to Hsp40, leading to restoration of wild-type p53 conformation and tumor suppression of cancer cells carrying p53^{R175H} (37). However, CTM is also known to inhibit the hypoxia-inducible factor pathway by blocking the interaction of HIF proteins and their cofactor p300. Moreover, it suppresses *in vivo* tumor growth of HCT116 (wild-type p53) cells and enhances radiosensitivity of cancer cells regardless of the p53 status (61–63). Thus, CTM has other function than mutant p53 reactivation.

PK7088

PK7088 [1-methyl-4-phenyl-3-(1H-pyrrol-1-yl)-1H-pyrazole] was identified as a compound that binds to a p53^{Y220C}-specific surface cavity destabilizing this protein through protein-observed NMR screening (38, 64). PK7088 stabilizes p53^{Y220C} and restores wild-type p53 conformation. It is biologically active in cancer cells carrying p53^{Y220C} mutant and induces G2/M arrest of the cell cycle and apoptosis with upregulation of NOXA and p21, as well as relocation of BAX to the mitochondria (38). PK7088 and Nutlin-3a cooperatively upregulate protein expression of p21 and NOXA (38). Crystal structure of p53-Y220C core domain with PK7242, a more soluble PK7088 analog, is also presented (38).

Stictic Acid (NSC87511)

A computational analysis of p53 structural models suggests that cysteine 124 of p53 is located at the center of a transiently open

binding pocket between loop L1 and sheet S3 of the p53 core domain (39). Based on the finding that additional mutation at cysteine 124 to alanine on $p53^{R175H}$ ($p53^{C124A/R175H}$) abolished the effects of PRIMA-1 on the reactivation of $p53^{R175H}$, Wassman et al. (39) performed an Ensemble-based virtual screening against this pocket and identified stictic acid as a p53 reactivation compound (39). Stictic acid stabilizes p53 *in vitro* and induces expression of p21 and PUMA in Saos2 (p53-null) cells expressing mutant p53 ($p53^{R175H}$ and $p53^{G245S}$) (39).

P53R3

The p53 reactivator (P53R3) is a compound identified through a screening using an *in vitro* DNA-binding assay (40). P53R3 restores sequence-specific DNA binding of several p53 mutants ($p53^{R175H}$, $p53^{M237I}$, and $p53^{R273H}$) and induces p53-dependent antiproliferative effects with increase in mRNA expression of many p53 target genes, such as *p21*, *GADD45*, *BAX*, *PUMA*, *PIG3*, and *MDM2* (40). It should be noted that P53R3 also increases mRNA expression of several p53 target genes (*p21*, *GADD45*, *PUMA*, and *MDM2*) in cancer cells expressing wild-type p53 (40).

SCH529074

The small molecule SCH529074 was identified by a DNA-binding assay-based screening as a compound that enabled $p53^{R273H}$ to bind to a consensus p53 DNA-binding site (41). SCH529074 restores the PAb1620 epitope by acting as a chaperone and enhances DNA-binding activity of several p53 mutants ($p53^{R175H}$, $p53^{S241F}$, $p53^{R248W}$, $p53^{R249S}$, and $p53^{R273H}$), leading to upregulation of p53 downstream target genes (*p21*, *BAX*, *NOXA*, *cyclin G1*, and *PUMA*), induction of proliferation arrest or apoptosis, and inhibition of *in vivo* tumor growth of mutant p53-expressing cell lines (41). Additionally, SCH529074 binds to DNA-binding domain of p53 and inhibits ubiquitination and degradation of wild-type p53 by MDM2 (41).

WR-1065

WR1065 is an active form of amifostine and is used to protect tissues against the damaging effects of radiation and chemotherapeutic drugs (42). WR-1065 increases wild-type p53 activity through a JNK-dependent signaling pathway, but not through genotoxic mechanisms (42–44). It is also reported that WR-1065 restores wild-type p53 conformation of a temperature-sensitive $p53^{V272M}$, leading to increase in the DNA-binding activity, transactivation of several p53 target genes (*p21*, *GADD45*, and *MDM2*), and cell cycle arrest in G1 phase (45).

COMPOUNDS THAT DEPLETE MUTANT p53

Although many p53-reactivating compounds seem to target more than one p53 mutant, it remains unclear if these compounds can reactivate all p53 mutants or specific mutant types. Also, PRIMA-1^{MET} is the only drug currently under clinical trials. Thus, the development of p53-reactivating compounds remains challenging. Another approach to target oncogenic mutant p53 is to discover compounds that specifically deplete mutant p53 with little effect on wild-type p53. Rationale to develop mutant p53-depleting compounds is based on the following observations: (1) mutant p53 is inherently unstable, but once stabilized, it can accelerate tumor progression (10) and (2) knockdown of mutant p53 by small interference RNAs (siRNAs) or shRNAs reduces malignant properties of cancer cells (13–16), thus indicating that oncogenic potential of cancer cells are, at least partially, dependent on the presence of accumulated oncogenic mutant p53. Although the mechanisms behind stabilization or degradation of mutant p53 are not necessarily the same as those of wild-type p53 and remain elusive (5, 65), several compounds that induce mutant p53 degradation without altering wild-type p53 have been found. These compounds can be used as effective therapeutic strategies for both cancers carrying only mutant p53 and those retaining wild-type p53 with mutant p53 as mentioned in the Introduction. Thus, compounds that specifically deplete mutant p53 are valuable for cancer therapy and also for elucidating the mechanisms of stabilization or degradation of mutant p53. Major compounds which deplete mutant p53 are listed in **Table 2** and explained below.

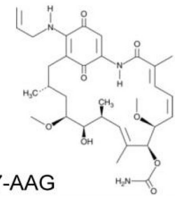
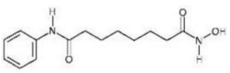
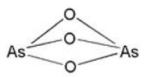
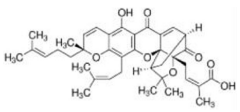
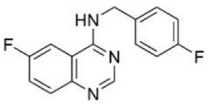
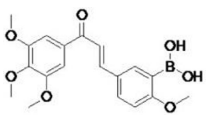
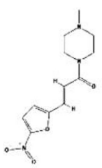
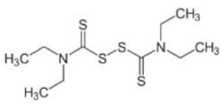
Hsp90 Inhibitors: Geldanamycin, 17-AAG, Ganetespib

Blocking the function of heat shock protein 90 (Hsp90) leads to depletion of several oncogenic proteins, such as Raf-1, ErbB2, and mutant p53 (66, 67), because Hsp90 contributes to accumulation of mutant p53 by inactivating p53 ubiquitin ligases, MDM2, and CHIP (68, 69). Treatment of cancer cells with 17-AAG, a Hsp90 inhibitor and an analog of geldanamycin, promotes degradation of varieties of p53 mutants ($p53^{R175H}$, $p53^{L194F}$, $p53^{R273H}$, and $p53^{R280K}$) and decreases viability of cells carrying mutant p53 (69). Importantly, another Hsp90 inhibitor, ganestespib, which is 50-fold more potent than 17-AAG in destabilizing mutant p53 with little effect on wild-type p53 levels, induces mutant p53 depletion with increased apoptosis in tumors *in vivo* in both $p53^{R248Q}$ Hupki (human p53 knock-in) and $p53^{R172H}$ knock-in mouse models (13). Ganetespib is currently under evaluation in clinical trials, including phase III for lung cancer (81–83).

Histone Deacetylase Inhibitors: Vorinostat/SAHA, Romidepsin/Depsipeptide

The effects of histone deacetylase inhibitors (HDACi) on mutant p53 ($p53^{R175H}$, $p53^{R280K}$, and $p53^{V274F/P223L}$) were first reported by Blagosklonny et al. (70). Later, two mechanisms describing the inhibition of HDAC8-mediated mutant p53 transcription (84) and mutant p53 destabilization through inhibition of HDAC6 by HDACi are proposed (71). Specifically, suberoylanilide hydroxamic acid (SAHA, also known as vorinostat), a FDA-approved HDACi that inhibits class I, II, and IV HDACs, induces degradation of mutant p53 by inhibiting HDAC6 activity, an essential positive regulator of Hsp90, and subsequent disruption of the HDAC6/Hsp90/mutant p53 complex, leading to mutant p53 ubiquitination by MDM2 and CHIP (71, 72). SAHA shows higher cytotoxic effects on cancer cells carrying mutant p53 than those having wild type or null for p53 (72). SAHA also sensitizes cancer cells to a topoisomerase inhibitor camptothecin in a mutant p53-dependent manner (71).

TABLE 2 | Compounds that deplete mutant p53.

Compound	Type of mutant	Mechanism	Reference	Structure
Hsp90 inhibitors: 17-AAG, geldanamycin, ganetespib	R175H, L194F, R248Q, R273H, R280K, R172H (mouse)	Reverse the Hsp90's function to inactivate MDM2 and CHIP	(13, 66–69)	 17-AAG
HDAC inhibitors: vorinostat/ SAHA, romidepsin/ depsipeptide	R175H, R280K, V247F/P223L	Inhibit HDAC6 and disrupt the HDAC6/ Hsp90/mutant p53 complex	(70–72)	 SAHA
Arsenic compounds	R175H, R248W, H179Y/R282W, R273H	Increase transcripts of Pirh2 and induce degradation of mutant p53	(73, 74)	 Arsenic trioxide
Gambogic acid	R175H, G266E, R273H, R280K	Inhibit the mutant p53-Hsp90 complex and induce CHIP-dependent degradation	(75)	
Spautin-1	R158lnF, R175H, S241F, R248Q, G266Q, R280L, R273H	Induce mutant p53 degradation via the CMA pathway activated by the suppression of macroautophagy under glucose-free and confluent conditions	(76, 77)	
YK-3-237	V157F, M237I, R249S, R273H, R280K	Decrease mutant p53 levels through deacetylation at lysine 382 by activating SIRT1	(78)	
NSC59984	R175L, R175H, S241F, R273H/ P309F	Induce MDM2-mediated mutant p53 degradation and activate p73	(79)	
Disulfiram (DSF)	R273H	Induce degradation of both wild-type p53 and $p53^{R273H}$ via the 26S proteasome pathway	(80)	

Arsenic Compounds

Arsenic trioxide (ATO), which is used to treat patients with acute promyelocytic leukemia (APL), binds to thiol groups in cysteine residues and induces degradation of proteins, such as promyelocytic leukemia protein (PML) and PML-retinoic acid receptor α (PML-RARα) fusion protein (85). It also activates wild-type p53 and upregulates p53 downstream target genes with induction of apoptosis (86). Yan et al. (73) asked the possibility of using arsenic compounds to target mutant p53 for degradation and found that ATO or sodium arsenite induced proteasomal-dependent degradation of several p53 mutants ($p53^{R175H}$, $p53^{H179Y/R282W}$, $p53^{R248W}$, and $p53^{R273H}$). ATO also increases transcripts of an E3 ubiquitin ligase Pirh2, leading to ubiquitination and degradation of several mutant p53 (74). However, it should be noted that arsenic compounds have carcinogenic effects and are known to induce several types of cancer (87).

Gambogic Acid

Gambogic acid (GA), a natural product derived from Garcinia hanburyi tree, induces apoptosis and inhibits tumor growth *in vivo* by upregulating wild-type p53 at protein levels (88). On the other hand, GA induces nuclear exports of mutant p53 ($p53^{R175H}$, $p53^{G266E}$, $p53^{R273H}$, and $p53^{R280K}$) for degradation by CHIP ubiquitin ligase (75). GA prevents the mutant p53-Hsp90 complex formation but enhances the mutant p53-Hsp70 interaction (75). Biologically, GA reduces viability of mutant p53-expressing cancer cells and also increases cytotoxic effects of several

chemotherapy drugs in human breast cancer MDA-MB-435 ($p53^{G266E}$) cell line (75).

Spautin-1

Spautin-1 is a derivative of MBCQ (4-((3,4-methylenedioxybenzyl)amino)-6-chloroquinazoline) which was identified as a small molecule that inhibited autophagy through an imaging-based screen using LC3-GFP as a marker (89). When cancer cells are placed in the nutrient-deficient environment, cells start autophagy to generate an alternative energy source to survive by degrading cellular proteins and organelles in the lysosome. Spautin-1 inhibits ubiquitin-specific peptidases, USP10 and USP13, and promotes the degradation of Vps34-PI3 kinase complexes, key regulators of autophagy, leading to inhibition of autophagy (89). Since USP10 also deubiquitinates wild-type p53, Spautin-1 promotes degradation of wild-type p53 (89, 90). Moreover, suppression of macroautophagy by Spautin-1 under glucose-free and confluent conditions is found to induce degradation of several p53 mutants ($p53^{R158InF}$, p53R175H, $p53^{R248Q}$, $p53^{S241F}$, $p53^{G266E}$, $p53^{R280L}$, and $p53^{R273H}$) through the chaperone-mediated autophagy (CMA) pathway (76). Spautin-1 also induces cell death under non-proliferating condition only when cancer cells express mutant p53. This effect of Spautin-1 is independent of MDM2 and the ubiquitin–proteasome pathway, but is dependent on nuclear export of mutant p53 and the presence of Hsc70 (76, 77).

YK-3-237

YK-3-237 was originally identified as a compound that showed antiproliferative effects in different cancer cell lines, but its mechanism of action was unknown (91). Yi et al. (78) investigated the effects of this compound on the proliferation of breast cancer cell lines carrying different p53 status and found that YK-3-237 preferentially inhibited the proliferation of breast cancer cell lines carrying mutant p53. YK-3-237 also decreased the levels of mutant p53 ($p53^{V157F}$, $p53^{M237I}$, $p53^{R249S}$, $p53^{R273H}$, and $p53^{R280K}$) through reduction in acetylation at lysine 382 (K382) of mutant p53, a target site of a NAD^+-dependent protein deacetylase SIRT1 (also known as sirtuin 1) (78). Indeed, YK-3-237 activates SIRT1 enzyme activity (78). Furthermore, treatment of triple-negative breast cancer cell lines with YK-3-237 results in induction of apoptosis and G2/M arrest of the cell cycle with increase in mRNA expression of *NOXA* and *PUMA* (78). However, the underlying mechanism remains unclear.

NSC59984

The small molecule NSC59984 was recently identified as a compound that increased luciferase activity in SW480 ($p53^{R273H/P309S}$) and DLD-1 ($p53^{S241F}$) cells carrying a p53-responsive luciferase reporter construct (PG13) (79). NSC59984 increases mRNA and protein levels of several p53 targets, such as p21, PUMA, and NOXA, with increase in apoptosis. It should be noted that NSC59984 also induces apoptosis in cancer cells having wild type and null for p53, suggesting it has p53-independent effects on cell death (79). Moreover, NSC59984 induces degradation of several p53 mutants ($p53^{R175L}$, $p53^{R175H}$, $p53^{S241F}$, and $p53^{R273H/P309S}$) through MDM2-mediated ubiquitination, whereas it rather increases wild-type p53 levels (79). Importantly, the effects of NSC59984 on p53 target gene expression and apoptosis are caused by activation of p73, rather than conformational changes of mutant p53 (79). In *in vivo* xenograft mouse models, NSC59984 suppresses tumor growth of DLD-1 in a p73-dependent manner (79).

Disulfiram

Disulfiram (DSF) is used for the treatment of chronic alcoholism by inhibiting acetaldehyde dehydrogenase. DSF has also been under clinical trials for some types of cancer including glioblastoma multiforme and metastatic non-small cell lung cancer (92, 93). DSF and its metabolites which are strong ROS inducers contain a reactive disulfide bond and readily mediate thiol-conjugating reactions, leading to S-glutathionylation of cysteine residues in proteins (94, 95). Protein S-glutathionylation in response to oxidative stress can affect function and stability of target proteins (80, 96). Interestingly, p53 is found to be S-glutathionylated at cysteine residues 124 and 141 (97, 98). Paranjpe et al. (80) reported that DSF and its derivative copper-chelated disulfiram (CuDSF) induced degradation of both wild-type p53 and $p53^{R273H}$ through the 26S proteasome pathway. However, DSF-induced protein degradation is not p53-specific, because it also induces degradation of other redox-regulated proteins, such as NF-κB subunit p50 and UBE1 (80).

OTHER STRATEGIES TO TARGET MUTANT p53 AND ITS RELATED GOF ACTIVITY

Compounds That Induce Readthrough of Premature Termination Codons

About 8% of all the p53 mutations in human cancers are nonsense mutations, which results in the presence of premature termination codons (PTCs) (99). PTC leads to the mRNA degradation by the nonsense-mediated mRNA decay pathway or potential production of truncated proteins. However, aminoglycoside antibiotics (gentamicin, G418, and amikamicin) bind with the ribosomal RNA and promote readthrough of PTCs, leading to partial restoration of full-length protein production (100). Specifically, gentamicin, G418, and NMDI14 induce production of full-length functional p53 from $p53^{Q192X}$, $p53^{R213X}$, and $p53^{E298X}$, leading to apoptosis induction in cancer cells carrying nonsense p53 mutations (101, 102).

Knockdown with Small Interference RNA

Knocking down specific protein expression by siRNAs or shRNAs can be a specific and potent strategy to target cancers if methodologies of efficient *in vivo* delivery are established (103). Indeed, downregulation of mutant p53 in T47D ($p53^{L194F}$), MDA-MB-231 ($p53^{R280K}$), and MDA-MB-468 ($p53^{R273H}$) breast cancer cell lines induces PARP-dependent apoptosis (104, 105). In DU145 ($p53^{P223L/V274F}$) prostate cancer cell line, downregulation of $p53^{P223L/V274F}$ by siRNA induces cell cycle arrest at G1 and G2/M phases, as well as apoptosis in a PI3K/Akt-dependent manner (106). Also, silencing of mutant p53 in 5637 ($p53^{R280T}$) and T24 (in-frame deletion of Y126) bladder cancer cell lines by

p53 siRNA induces G2 arrest of the cell cycle and apoptosis, and increases sensitivity to cisplatin (107). Thus, accumulated studies reveal that knockdown of mutant p53 by siRNAs or shRNAs reduces malignant properties of cancer cells. However, siRNAs for p53 used in aforementioned publications can also knockdown wild-type p53. Matinez et al. (108) first developed a $p53^{R248W}$-specific siRNA which did not affect wild-type p53. Recently, our laboratory also developed siRNAs specific for $p53^{R175H}$ and $p53^{R273H}$, which did not alter wild-type p53 expression levels (unpublished). We successfully showed reactivation of wild-type p53 and reduced cell proliferation and migration, following transfection of these mutant-specific siRNAs in genetically engineered *p53* heterozygous cancer cell lines ($HCT116^{+/R248W}$, $SW48^{+/R273H}$) (unpublished). Thus, the extraordinary sequence specificity of siRNA makes it an attractive tool for targeted cancer therapies.

Compounds That Affect Downstream Targets of Mutant p53

Another way to target oncogenic activity of mutant p53 is to reactivate tumor suppressive pathways that are inhibited by mutant p53 or to inhibit tumor-promoting pathways that are activated by mutant p53.

Reactivate transcriptional activity (RETRA) was identified as a compound that increased β-galactosidase activity in A431 ($p53^{R273H}$) human epidermal carcinoma cells expressing a p53-resonsive promoter-driven β-galactosidase construct, through high-throughput screening of a chemical library comprising of 46,250 compounds (109). RETRA increases β-galactosidase activity only in cancer cells carrying mutant p53 ($p53^{R248Q}$, $p53^{R280L}$, and $p53^{G266E}$) with increased mRNA expression of *p21* and *PUMA*, but fails to do so in cells wild type or null for p53. Interestingly, the effects of RETRA are nullified by knockdown of p73, but not knockdown of p53 and p63 (109). Indeed, RETRA inhibits the binding of $p53^{R273H}$ with p73. RETRA also reduces A431 cell viability in a p73-dependent manner and reduces tumor growth in a xenograft model (109).

Statins are a class of compounds that inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in the mevalonate pathway and have been used in the clinic to treat hypercholesterolemia. HMG-CoA reductase is the rate-limiting step in cholesterol synthesis and also regulates prenylation/lipidation (farnesylation and geranyl-geranylation) of proteins. Prenylation facilitates attachment of target proteins to cell membranes which are involved in cellular adhesion, migration, and proliferation signaling (e.g., Rho, Rac, Cdc42, Ras) (110). Interestingly, knockdown of $p53^{R273H}$ significantly reduces mRNA expression of multiple enzymes involved in the mevalonate pathway (16). Both knockdown of mutant p53 and inhibition of protein prenylation by statins or other compounds result in impaired growth of breast cancer cells in 3D culture (16). Mechanistically, mutant p53 binds to and activates SREBP, crucial transcription factors that regulate transcription of several enzymes involved in the mevalonate pathway, leading to enhanced prenylation of proteins associated with cancer progression and activation of prenylated proteins in breast cancer cells; hence, inhibition of protein prenylation by statins leads to reduced malignancy of human breast cancer cells (16). Importantly, the presence of p53 mutation correlates with high expression of sterol biosynthesis genes in human breast tumors (16). Additionally, since nuclear localization and activation of the YAP and TAZ proto-oncogenes are regulated by prenylation and activation of Rho GTPases, statins could also suppress progression of mutant p53-expressing tumors by inhibiting YAP/TAZ activation by reducing protein prenylation of Rho GTPases, which is promoted by SREBP and its cofactor mutant p53 (111).

Compounds That Induce Synthetic Lethality

Synthetic lethality is generally used for the condition where a mutation in a gene is not lethal by itself, but its combination with a drug or other gene mutations leads to cell death (112). Since over 50% of human cancers have mutations in the *p53* gene, p53 mutations become attractive targets for inducing synthetic lethality in tumors. In this regard, compounds that induce synthetic lethality to mutant p53 should selectively kill cancer cells expressing mutant p53 without affecting normal cells carrying wild-type p53. One compound that induces synthetic lethality to mutant p53 is UCN01, a protein kinase C inhibitor and a potent blocker of G2/M checkpoint of the cell cycle. Treatment of CA46 ($p53^{R248Q}$) and HT29 ($p53^{R273H}$) with UNO01 abrogates γ-irradiation-induced G2/M arrest of the cell cycle and increases cytotoxicity (113). UCN01 also enhances cisplatin-induced cell death in MCF7-expressing human papillomavirus type-16 E6 (MCF7/E6) with little effect on parental MCF7 cells having functional p53 (113). Another compound that is synthetic lethal to mutant p53 is BI-2536, an inhibiter of polo-like kinase 1 (PLK1), an enzyme that controls G2/M checkpoint. Inhibition of PLK1 by BI-2536 significantly enhances cytotoxic effect of ionizing radiation in DLD-1 ($p53^{S241F}$) and $p53^{R248W}$-overexpressing HCT116 cells, but it does not do so in parental HCT116 (wild-type p53) cells (114). Similar effects are observed with PD0166285, an inhibitor of Wee1 kinase that regulates G2/M checkpoint. PD0166285 sensitizes cancer cells (HT29: $p53^{R273H}$ and E6-overexpressing PA-1: wild-type p53) to radiation-induced death, whereas this effect is not detected in parental PA-1 cells (115). However, the observed synthetic lethality to mutant p53 is likely not dependent on oncogenic GOF activity of mutant p53, but rather dependent on loss of wild-type p53 activity. Identification of synthetic lethality to mutant p53 alone, but not p53-null, could improve our understanding of oncogenic GOF activity of mutant p53 and contribute to the development of new compounds that target cancer cells carrying mutant p53.

SUMMARY AND FUTURE DIRECTIONS

Accumulated evidence has proven that small-molecule compounds can restore the transcriptional activity of mutant p53 or specifically deplete mutant p53. These compounds are expected to efficiently inhibit tumor growth with minimal effects on normal tissues. Several compounds listed in **Tables 1** and **2** are already in clinical trials. Within p53 reactivators, $PRIMA\text{-}1^{MET}$ (also known as APR-246) is the only drug under clinical trials (51, 52). On the other hand, amongst the compounds that deplete mutant p53, Hsp90 inhibitors (81–83), HDAC inhibitors (116–118), ATO (119–121),

and DSF (92, 93, 122) are under clinical trials for cancer therapy. However, it is not completely understood whether these p53 reactivators and mutant p53-depleting compounds have mechanisms of action on proteins or pathways other than mutant p53, if they have an impact on all p53 mutants or specific mutant types and their underlying mechanisms. Also, it would be important to determine any synergistic or additive effects of these compounds with conventional chemotherapy drugs on cancer cell survival and proliferation. Further studies to solve these concerns would help improving the efficacy and specificity of these compounds.

In order to better understand the mechanisms of action of compounds that target mutant p53, it is important to determine whether they can directly bind with mutant p53 or proteins involved in the process of mutant p53 reactivation or depletion. There are several *in vitro* methodologies to determine compound–protein interaction, including Biacore assays, mass spectrometry-based approaches, and drug affinity responsive target stability (DARTS) assays (123–125). On the other hand, *in vivo* analysis is limited. Recently, Jafari et al. (126) reported a cell-based drug–protein interaction assay, called cellular thermal shift assay (CETSA). Investigating interactions between a compound and a specific protein in cells would significantly improve our understanding of how efficiently and specifically the compound alters intracellular activity.

Biological effects of compounds that restore p53 activity are robust, since mutant p53 is usually accumulated in cancer cells, and hence these compounds have ample substrates to restore the p53 activity. On the other hand, compounds that deplete mutant p53 may not be as robust as those restoring the p53 activity; however, as mentioned in Section "Introduction," survival and growth of cancer cells are frequently dependent on mutant p53 (oncogene addiction) (13, 14). Thus, simply depleting mutant p53 in tumors is likely sufficient to reduce tumor malignancy. Especially, when tumor cells retain the wild-type *p53* allele, compounds depleting mutant p53 alone may be even more effective, since they could reactivate wild-type p53 by releasing from DN activity of mutant p53. These mutant p53-depleting compounds could also be used for prevention of tumorigenesis when patients inherently carry mutant p53, such as in the case of human tumor-prone disease Li–Fraumeni syndrome (LFS). Over 70% of LFS patients have p53 mutations in their germlines (127), but mutant p53 is not always stable and accumulates mainly in tumors (10). A compound that depletes only accumulated mutant p53 in tumors could reduce risk of tumor development in LFS patients. The approach of mutant p53-specific knockdown by siRNAs or DNA oligomers could cause similar outcomes to those by mutant p53-depleting compounds. However, the major hurdle of this approach is efficient delivery of these oligomers to all cancer cells, which need to be addressed prior to their consideration for clinical trials (103).

Other than compounds summarized in this review article, compounds that target inhibitors of p53, such as MDM2 and MDM4, are also powerful to reactivate p53 and are summarized in other review articles (128, 129). Also, induction of synthetic lethality for mutant p53 is another specific approach for cancer cells expressing mutant p53 (128, 130–132). Inhibitors of proteins associated with G2/M arrest of the cell cycle and mitotic checkpoint appear to cause mitotic catastrophe in cancer cells lacking wild-type p53 activity.

In summary, discovering efficient and safe compounds that specifically target mutant p53 remains challenging. Hence, it is crucial to further understand how mutant p53 induces oncogenic function, to elucidate the exact mechanisms of mutant p53 stabilization or degradation in tumors, and to identify mutant p53-specific downstream signaling pathways or binding partners. The battle with cancer is unexpectedly taking longer, with cancers being wily enough to escape from current available treatments and develop novel ways of surviving. Given that several mutant p53-targeting drugs are already undergoing clinical trials, the goal toward establishment of therapies to cure mutant p53-carrying cancers may be just on the horizon.

AUTHOR CONTRIBUTIONS

AP and TI wrote the manuscript.

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REVIEW
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Targeting of Mutant p53 and the Cellular Redox Balance by APR-246 as a Strategy for Efficient Cancer Therapy

*Vladimir J. N. Bykov[1], Qiang Zhang[1], Meiqiongzi Zhang[1], Sophia Ceder[1], Lars Abrahmsen[2] and Klas G. Wiman[1]**

[1] Department of Oncology-Pathology, Cancer Center Karolinska (CCK), Karolinska Institutet, Stockholm, Sweden, [2] Aprea AB, Solna, Sweden

TP53 is the most frequently mutated gene in cancer. The p53 protein activates transcription of genes that promote cell cycle arrest or apoptosis, or regulate cell metabolism, and other processes. Missense mutations in TP53 abolish specific DNA binding of p53 and allow evasion of apoptosis and accelerated tumor progression. Mutant p53 often accumulates at high levels in tumor cells. Pharmacological reactivation of mutant p53 has emerged as a promising strategy for improved cancer therapy. Small molecules that restore wild type activity of mutant p53 have been identified using various approaches. One of these molecules, APR-246, is a prodrug that is converted to the Michael acceptor methylene quinuclidinone (MQ) that binds covalently to cysteines in p53, leading to refolding and restoration of wild type p53 function. MQ also targets the cellular redox balance by inhibiting thioredoxin reductase (TrxR1) and depleting glutathione. This dual mechanism of action may account for the striking synergy between APR-246 and platinum compounds. APR-246 is the only mutant p53-targeting compound in clinical development. A phase I/IIa clinical trial in hematological malignancies and prostate cancer showed good safety profile and clinical effects in some patients. APR-246 is currently tested in a phase Ib/II trial in patients with high-grade serous ovarian cancer.

Keywords: APR-246, mutant p53, apoptosis, thioredoxin reductase, glutathione, redox balance, clinical trial, cancer therapy

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****Correspondence:***
Klas G. Wiman
klas.wiman@ki.se

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INTRODUCTION

Recent DNA sequencing of 3281 human tumors within The Cancer Genome Atlas (TCGA) has confirmed the high frequency of TP53 mutations in cancer. At least 42% of the cases of 12 common human tumor types carry mutant TP53 (1). In high-grade serous (HGS) ovarian cancer, the fraction of tumors with mutant TP53 is almost 95%. No other gene is mutated at such high frequency in cancer. See also TP53 databases p53.iarc.fr and p53.free.fr. The second and third most frequently mutated genes are PIK3CA that encodes the p110 alpha catalytic subunit of PI3 kinase and PTEN, a lipid phosphatase that regulates Akt kinase activation, which are mutated in 17.8 and 9.7% of the cases of the 12 common tumor types, respectively (1).

Wild type p53 protein induces cell cycle arrest, senescence, and apoptosis in response to cellular stress by upregulating target genes such as p21, Bax, Puma, and Noxa (2). p53 can also regulate cell

metabolism and redox status through target genes such as TIGAR and GLS2 (3–5). It remains unclear exactly how p53 mediates potent tumor suppression. *In vivo* studies in mice have shown that certain engineered p53 mutants that fail to transactivate pro-arrest and pro-apoptosis target genes can still prevent tumor development (6, 7). Similarly, mice lacking the p53 target genes p21 that mediates p53-dependent cell cycle arrest and Puma and Noxa that mediate p53-dependent apoptosis do not show increased tumor incidence (8). These findings argue that other p53 transcriptional targets, for instance those involved in regulation of metabolism, are critical for p53-mediated tumor suppression.

Oncogenic stress as a result of oncogene activation or loss of cell cycle control characterizes early stages of tumor evolution. This leads to aberrant DNA replication, which triggers a DNA damage response (DDR) involving activation of ATM, Chk1 and Chk2 kinases, and p53, and induction of senescence or apoptosis (9). Activation of DDR and p53 upon oncogenic stress serves to eliminate incipient tumor cells and forms a critical barrier against tumor development. DDR inactivation by mutation in ATM or TP53 allows cell survival and tumor progression. Many TP53 mutations are missense mutations resulting in amino acid substitutions in the DNA-binding core domain and disruption of p53-specific DNA binding and transcriptional transactivation (10). Loss of wild type p53 is associated with increased resistance to chemotherapy.

The high frequency of missense TP53 mutations in human tumors and the fact that mutant p53 often accumulates at high levels in tumor cells make mutant p53 a potential target for improved cancer therapy. Pharmacological reactivation of mutant p53 would restore p53-dependent senescence and apoptosis, and presumably also p53-mediated regulation of metabolism and other processes, and thus eliminate tumors *in vivo*. Indeed, studies in various mouse models have demonstrated that restoration of wild type p53 expression *in vivo* leads to rapid tumor elimination (11–13). This suggests that restoration of functional p53 can trigger tumor cell death and lead to tumor clearance even if a tumor carries multiple genetic alterations that drive tumor growth.

PHARMACOLOGICAL REACTIVATION OF MUTANT p53

A growing number of small molecules that can reactivate mutant p53 have been identified over the past 15 years, using either chemical library screening or rational drug design. These include CP31398, PRIMA-1Met/APR-246, PK-083, PK-5174, SCH529074, and NSC319726 (ZMC1). We have previously reviewed this field (14). This review is focused on PRIMA-1 (APR-017) and the structural analog PRIMA-1Met, now named APR-246, both of which were identified in our laboratory. As will be discussed below, both compounds are prodrugs that form the active moiety MQ. We will also highlight the clinical development of APR-246.

We identified PRIMA-1 in a screen of a small structurally diversified chemical library from NCI (Diversity set) for compounds that could induce cell cycle arrest or cell death preferentially in cells expressing mutant p53 (15). Cell growth and viability were assessed by the WST1 assay. PRIMA-1 showed the strongest preference for mutant p53-expressing cells and was selected for further studies. Experiments with antibodies specific for correctly folded wild type p53 (PAb1620) or unfolded mutant p53 (PAb240) revealed that PRIMA-1 could induce refolding of mutant p53 and enhance mutant p53 DNA binding in gel shift assays. PRIMA-1 treatment of tumor cells carrying various mutant p53 resulted in upregulation of p53 target genes such as p21, Bax, and Mdm2, and induction of cell death by apoptosis. Systemic administration of PRIMA-1 in mice carrying Saos-2-His273 tumor xenografts demonstrated significant inhibition of xenograft tumor growth *in vivo* (15). In parallel, our analysis of available data in the NCI database confirmed that PRIMA-1 preferentially targets tumors cells carrying mutant p53 and has an activity profile that is entirely distinct from those of commonly used chemotherapeutic drugs such as cisplatin and 5-FU (16). Subsequently, the structural analog PRIMA-1Met or APR-246 that has superior permeability properties was identified. APR-246 was shown to synergize with chemotherapeutic drugs, e.g., adriamycin and cisplatin (17).

TARGETING MUTANT p53 BY MICHAEL ADDITION

Our data clearly showed that PRIMA-1 and APR-246 were able to reactivate various forms of mutant p53 and trigger tumor cell apoptosis, but their molecular mechanism of action remained obscure. However, we found that both compounds are converted to methylene quinuclidinone, MQ, a Michael acceptor that can react with soft nucleophiles such as thiols in proteins (**Figure 1**). The p53 core domain has 10 cysteine residues. Mass spectrometry demonstrated that MQ binds covalently to the p53 core domain (18). Several findings support the notion that MQ binding to p53 is critical for the effect of PRIMA-1 and APR-246. *N*-acetylcysteine (NAC), a thiol group donor, blocks PRIMA-1-induced apoptosis and PRIMA-D (APR-320), a structural analog that cannot be converted to MQ, has no effect on tumor cells at concentrations corresponding to those used for PRIMA-1 and APR-246. Moreover, transfer of MQ-modified mutant p53 protein into p53 null tumor cells induces expression of p53 target genes and cell death by apoptosis (18). These results demonstrate that MQ is the active compound and that MQ-modification of mutant p53 *per se* is sufficient to induce tumor cell death. Thus, PRIMA-1 and APR-246 are prodrugs that form the biologically active compound MQ (**Figure 1**). This conversion is spontaneous and occurs over a time frame of a few hours at physiological pH (18). Since MQ is reactive, its administration as a prodrug is probably critical in order to avoid adduct formation with various extracellular targets.

It is interesting to note that MIRA-1, another compound identified in our screen of the NCI Diversity set, is a maleimide with known Michael acceptor activity. Moreover, Kaar and Fersht and their colleagues identified a series of Michael acceptors that bind covalently to both wild type and mutant p53 core domains, resulting in increased protein melting temperature. Analysis of the reactivity of the cysteines in p53 by mass spectrometry

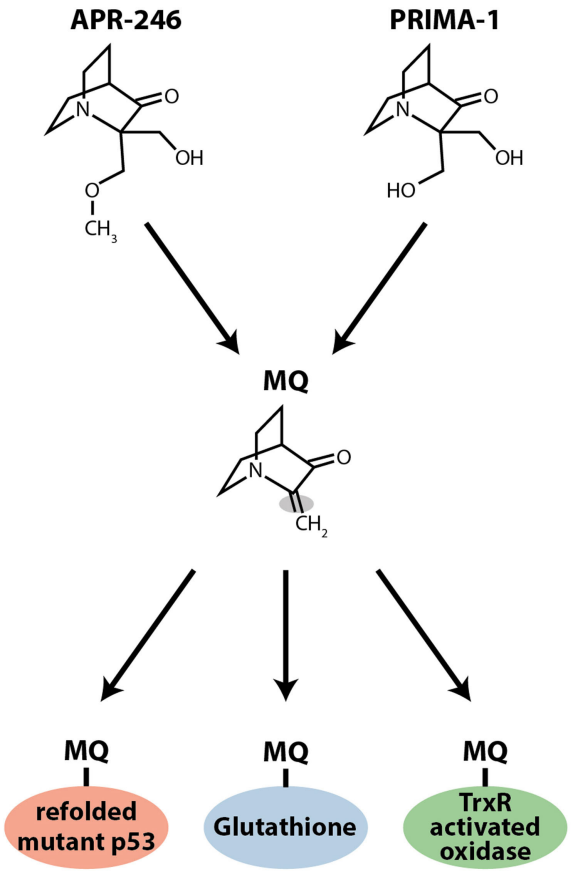

FIGURE 1 | Chemical structures of PRIMA-1 and APR-246 (PRIMA-1Met). Both compounds form the Michael acceptor methylene quinuclidinone (MQ), which is the active moiety. MQ binds covalently to thiols in mutant p53. MQ also targets thioredoxin reductase (TrxR) and glutathione (GSH). MQ binding to TrxR converts the enzyme to an active oxidase, which generates ROS, and MQ binding to glutathione depletes intracellular free glutathione, which also induces ROS.

revealed preferential reaction with C124 and C141, followed by C135, C182, and C277, and then C176 and C275 (19). These results further support the idea that adduct formation at cysteines can stabilize the native conformation of p53.

Among the 10 cysteine residues in p53's core domain, four – Cys182, Cys229, Cys242, and Cys277 – are exposed on the surface of the protein and accessible for modification in correctly folded p53 (20, 21). Presumably, additional cysteines are exposed in unfolded mutant (or wild type) p53, allowing more extensive thiol modification (18). Computational analysis of structural p53 models identified a binding pocket between the L1 loop and S3 sheet in the p53 core domain, containing cysteines C124, C135, and C141 (22). Docking analysis indicated that MQ, as well as other thiol-targeting compounds including MIRA-1, can bind to the L1/S3 pocket. These results were validated in living cells by introduction of a C124A substitution in R175H mutant p53. Indeed, C124A substitution abolished the apoptotic effect of PRIMA-1 in Saos-2 osteosarcoma cells expressing R175H mutant p53.

Thus, APR-246/MQ, MIRA-1 and the compounds identified by Kaar et al. (19) share a common chemical property and presumably promote refolding of mutant p53 by a similar mechanism. The ability to modify cysteines in mutant p53 distinguishes APR-246/MQ from compounds like PK-083 and PK-7088 that bind to a crevice in the Y220C mutant p53 protein and raise its melting temperature (23). APR-246 also has a different mechanism of action than the compound NSC319726 (ZMC1), a zinc chelator that refolds His175 mutant p53 as well as several other mutant p53 proteins (24). Clearly, mutant p53 refolding and reactivation can be achieved by various molecular strategies. Some strategies work for specific mutant forms of p53, whereas other strategies are applicable to a range of mutant p53 proteins.

APR-246 REACTIVATES MUTANT FORMS OF p53 FAMILY MEMBERS p63 AND p73

p53 is a member of a protein family with two other members, p63 and p73 (25). In contrast to TP53, neither the TP63 nor TP73 genes are mutated at any significant frequency in human tumors. However, TP63 missense mutations occur in certain developmental syndromes such as the Ectrodactyly–ectodermal dysplasia–cleft (EEC) syndrome (26). All three proteins share a high degree of sequence similarity in the DNA-binding core domain (25). The 10 cysteines in the p53 core domain are all conserved in both p63 and p73. This raises the question as to whether APR-246 can affect mutant p63 and/or p73 folding and activity. We first examined the effect of APR-246 on human tumor cells carrying exogenous temperature-sensitive missense mutant TP63 and TP73. APR-246 induced the expression of p53/p63/p73 target genes, cell cycle arrest, and cell death by apoptosis in these cells (27). To assess the effect of APR-246 on mutant p63 in a more physiological context, we used human keratinocytes derived from EEC patients carrying R204W or R304W mutant TP63. These two TP63 mutants correspond to the tumor-associated hot spot TP53 R175H and R273H mutants. Treatment with APR-246 led to increased expression of p63 target genes and at least a partial rescue of keratinocyte differentiation (28). Similarly, APR-246 rescued corneal differentiation in iPS cells from EEC individuals (29). Thus, the targeting of mutant versions of the two structurally related transcription factors p63 and p73 by APR-246 leads to entirely different biological responses that recapitulate the normal functions of each protein. These results argue convincingly that the biological effects of APR-246 are mediated by direct binding to mutant p53 or p63 and refolding the mutant proteins into an active conformation.

APR-246/MQ TARGETS COMPONENTS OF THE CELLULAR REDOX SYSTEM

The observation that MQ can bind to thiols suggested that it might also target thiol-containing redox regulators such as glutathione and thioredoxin. Indeed, we found that APR-246 is a potent inhibitor of thioredoxin reductase (TrxR1), a selenocysteine-containing enzyme that catalyzes the reduction of thioredoxin (30). APR-246 inhibits the activity of TrxR1 both *in vitro* and in

living cells. This effect is presumably mediated through modification of the selenocysteine residue in TrxR1 by MQ. MQ binding converts TrxR1 into an NADPH oxidase that contributes to ROS production and cell death induced by APR-246 (30).

Methylene quinuclidinone has also been shown to bind to cysteines in glutathione (GSH), leading to a decrease in free intracellular glutathione concentrations and increased ROS levels (31, 32). Since glutathione can mediate resistance to platinum drugs by conjugation and export, this effect of MQ may at least in part account for the strong synergy between APR-246 and platinum drugs (see below). APR-246 did not inhibit GCLM (regulatory subunit of γ-glutamyl cysteine-synthase) or GSS (glutathione synthetase) in the GSH synthesis pathway, indicating that the observed GSH depletion is not caused by decreased synthesis (32).

Thus, accumulating data on the effects of PRIMA-1/APR-246 on the cellular redox balance demonstrate that these compounds have a dual mechanism of action that targets two Achille's heels of tumor cells: mutant p53 and the redox balance (**Figure 2**). The targeting of these two pathways may allow more efficient elimination of tumor cells and lower the probability of resistance development. This dual mechanism provides an explanation for reported mutant p53-independent effects of APR-246.

EFFECTS ON MUTANT AND WILD TYPE p53

Methylene quinuclidinone can bind to both wild type and mutant p53 (18), and it is conceivable that MQ binding can induce refolding of misfolded wild type p53 in tumor cells. However, available data so far indicate that APR-246/MQ has little toxicity in normal cells. Wild type p53 is expressed at low levels in most normal cells and tissues in the absence of stress, whereas many tumor cells express high levels of unfolded mutant p53. Also, normal cells have a higher capacity to cope with oxidative stress as compared to tumor cells (33). While MQ binding to mutant p53 can restore p53-dependent apoptosis (18), MQ binding to other cellular proteins may not necessarily have major effects on cell growth and survival, except for binding to TrxR and GSH and possibly other anti-oxidative proteins, as discussed above. The benign safety profile of APR-246 observed in the first clinical study (34) is consistent with the lack of major toxicity in normal cells.

Interestingly, the response of wild type and mutant TP53-carrying tumor cells to MQ is enhanced by hypoxia (35). Hypoxia (≤1% oxygen) increased the sensitivity of SKBR3 cells (R175H mutant TP53) to PRIMA-1 treatment. In MCF-7 cells (wild type TP53), chemical hypoxia induced by $CoCl_2$ led to accumulation of unfolded wild type p53, as assessed with the monoclonal antibody PAb240, and enhanced sensitivity to PRIMA-1. Presumably, this is due to MQ binding and refolding of unfolded "mutant-like" wild type p53 into an active conformation. The finding that hypoxia can potentiate the efficacy of PRIMA-1 has important clinical implications. Due to insufficient blood supply, rapidly growing tumors *in vivo* are often hypoxic, and it is conceivable that this could enhance the therapeutic efficacy of APR-246, both in wild type and mutant TP53-carrying tumors.

SYNERGY WITH CONVENTIONAL CHEMOTHERAPEUTIC DRUGS AND NOVEL EXPERIMENTAL DRUGS

A major hurdle for achieving efficient elimination of tumors and long-term cancer cure is the rapid development of therapy resistance. There are numerous mechanisms for such resistance, including enhanced DNA repair and increased efflux of chemotherapeutic drugs from the tumor cell (36). The problem of resistance is relevant not only for conventional chemotherapeutic drugs but also for targeted drugs, as exemplified by resistance development in CML upon treatment with the novel drug imatinib (Gleevec) that inhibits the BCR-ABL kinase (37). Therefore, it is important to explore possible synergies between APR-246 and conventional anticancer agents, novel targeted drugs, and experimental drugs.

The DNA damage caused by chemotherapeutic drugs such as cisplatin and doxorubicin induces tumor cell death to a large extent via wild type p53 activation and p53-induced apoptosis. Accordingly, tumor cells carrying mutant p53 or completely lacking p53 are often more resistant to conventional chemotherapy. This suggests that restoration of wild type p53 function by APR-246 might synergize with, for example, cisplatin. Indeed, we and others have demonstrated strong synergy between APR-246 and chemotherapeutic drugs such as cisplatin, 5-fluorouracil (5-FU), and doxorubicin in mutant p53-carrying lung, ovarian, and esophageal cancer cells (17, 31, 38, 39). Synergistic effects have also been observed *in vivo* upon systemic administration (17, 39).

There are several possible reasons for the observed synergy (**Figure 2**). First, as alluded to above, restoration of wild type function to mutant p53 by APR-246 might increase sensitivity to chemotherapeutic drugs that depend on wild type p53 for induction of efficient tumor cell apoptosis. Second, treatment with cisplatin, adriamycin, or 5-FU leads to accumulation of mutant p53 (17, 39), which is expected to enhance the effect of APR-246. Third, we and others found that APR-246, via MQ, depletes intracellular GSH levels (31, 32). Since formation of adducts with GSH and extracellular export is one mechanism of cisplatin resistance, MQ-mediated GSH depletion is likely to sensitize tumor cells to cisplatin. Fourth, MQ-mediated inhibition of TrxR and conversion of the enzyme to an active oxidase (30) should induce ROS levels, which will further enhance DNA damage and p53-dependent cell death. Inhibition of TrxR will also negatively affect the activity of ribonucleotide reductase, needed for providing deoxyribonucleotides for DNA replication and repair (40).

In contrast to the mutant p53-dependent synergy of APR-246 with cisplatin and 5-FU, the synergy between APP-246 and epirubicin was p53-independent in esophageal cancer cells (39). This could be due to the redox effects of APR-246, including inhibition of TrxR and/or GSH depletion. Cisplatin and 5-FU, but not epirubicin, induced the expression of mutant p53 (39). Synergy has also been observed between APR-246 and the experimental compound RITA in AML cells. This synergy could arise from increased levels of mutant p53 upon induction of DNA damage by RITA (41).

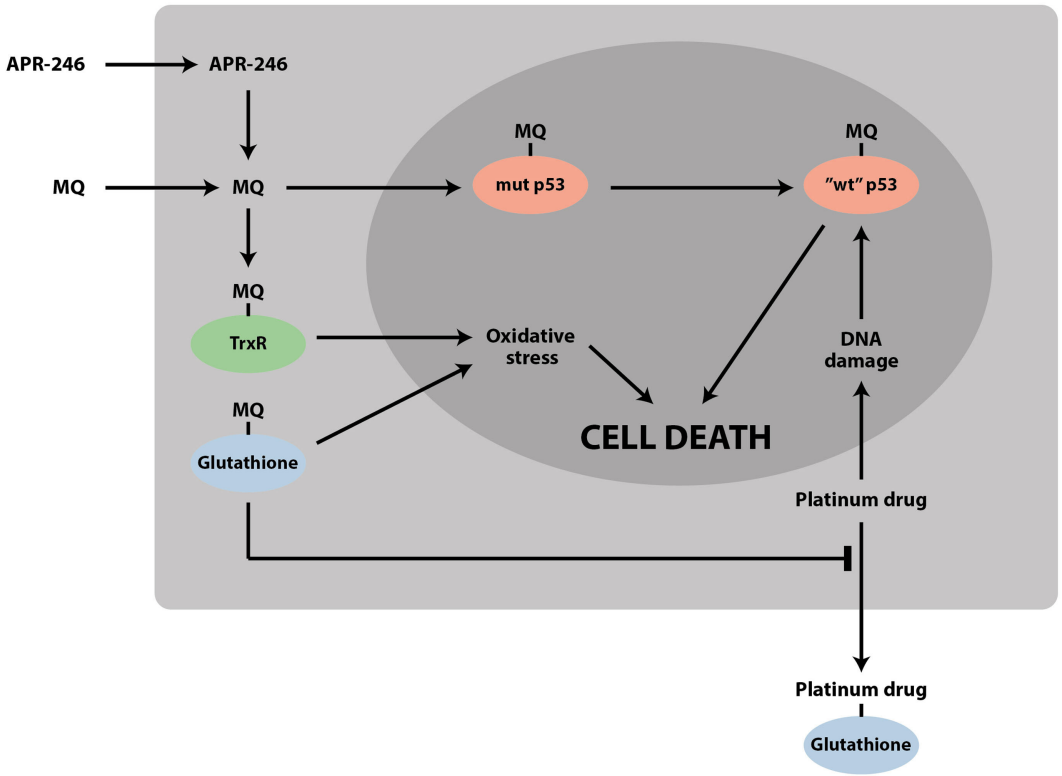

FIGURE 2 | APR-246/MQ targets both mutant p53 and the cellular redox balance. The striking synergy between APR-246 and DNA-damaging chemotherapeutic drugs such as cisplatin and doxorubicin can be due to (1) refolding and reactivation of mutant p53 by APR-246, which enhances the response to DNA-damaging agents; (2) accumulation of mutant p53 by DNA-damaging agents, which potentiates the effect of APR-246; (3) depletion of glutathione by MQ, which inhibits efflux of drugs such as cisplatin; and (4) inhibition of TrxR and conversion of the enzyme to an active oxidase, leading to increased oxidative stress.

In addition, APR-246 synergized with daunorubicin in AML cells carrying wild type p53 (41). Synergy in the absence of mutant p53 may be due to APR-246-mediated redox effects. However, as discussed above, MQ can also bind to wild type p53 and restore an active conformation under hypoxic conditions. There is evidence suggesting that wild type p53 may occur in a misfolded conformation in some tumors, e.g., B-CLL (42) and AML (43). This raises the possibility that refolding of wild type p53 by APR-246 may be responsible for synergy with chemotherapeutic agents in AML cells.

PRIMA-1 at 50 μM induced G2/M phase accumulation of parental mouse L1210 leukemia cells carrying mutant p53 but had only a minor effect on the cell cycle distribution of Y8 cells, a subline of L1210 that lacks p53. A striking synergistic induction of necrosis was observed in L1210 cells upon combination treatment with PRIMA-1 and the cyclin-dependent kinase inhibitor flavopiridol. However, in Y8 p53 null cells, combination of PRIMA-1 and flavopiridol caused a synergistic increase in apoptosis (44). Thus, combination treatment with PRIMA-1 (or presumably APR-246) can lead to cell death through alternative routes, depending on the presence or absence of mutant p53.

Mutant p53 reactivation by APR-246 leads to induction of the p53 target and antagonist Mdm2 (15), which promotes p53 degradation by the proteasome. Therefore, it is conceivable that inhibition of Mdm2-p53 binding and Mdm2-mediated p53 degradation might potentiate the effect of APR-246. Indeed, strong synergy was observed between PRIMA-1 and the Mdm2 inhibitor Nutlin-3 in pancreatic cancer cells (45). Moreover, gene therapy with the tumor suppressor gene FHIT (fragile histidine triad), whose gene product has been shown to inactivate Mdm2 (46), resulted in synergistic inhibition of tumor growth in combination with APR-246 (47). Since several compounds that disrupt p53-Mdm2 binding are now being tested in the clinic, these results may have profound implications for the future clinical use of both APR-246 and inhibitors of p53-Mdm2 binding.

CLINICAL DEVELOPMENT

APR-246 has been tested in a first-in-man phase I/IIa clinical trial in patients with hematological malignancies or hormone-refractory prostate cancer (34). The main aim was to determine maximum tolerated dose (MTD) of APR-246 and to assess safety and pharmacokinetic properties. Patients were not preselected based on TP53 mutation status. The treatment regimen was 2-h infusion of APR-246 for 4 days. Overall, the study showed that APR-246 is well tolerated and only relatively minor and transient side effects were observed, including dizziness, fatigue,

headache, nausea, and confusion. MTD was defined as 60 mg/kg. Plasma concentrations of APR-246 reached 250 μM, well above concentrations required for robust induction of tumor cell apoptosis in cell culture experiments. Analysis of isolated patient leukemic cells by FACS revealed induction of p53 targets Bax and Puma upon APR-246 treatment, and microarray analysis showed substantial alterations in gene expression, including genes associated with cell cycle regulation and cell death, consistent with the proposed mechanism of action. Furthermore, one AML patient carrying V173M mutant TP53 showed a significant reduction in bone marrow blasts, and one patient with a TP53 splice site mutation had a minor response according to CT scan. Thus, APR-246 is safe and shows signs of clinical activity. APR-246 is currently being tested in combination with carboplatin and pegylated doxorubicin in a phase Ib/II clinical study in HGS ovarian cancer, a tumor type with a 95% frequency of TP53 mutations (see www.clinicaltrials.gov).

FUTURE PERSPECTIVES

The development of efficient mutant p53-reactivating anticancer drugs is expected to have a major impact on public health globally, given the high frequency of TP53 mutations in a wide range of human tumors. In certain tumor types, TP53 is mutated in the great majority of the cases. In general, clinical studies have shown that mutant TP53-carrying tumors respond less well to conventional chemotherapeutic drugs and have worse prognosis than wild type TP53-carrying tumors. The ongoing phase Ib/II clinical study with APR-246 will provide solid data on clinical efficacy in combination with standard chemotherapy. Importantly, the mechanism of action of APR-246 – i.e., dual targeting of both mutant p53 and the cellular redox system – suggests that APR-246 will synergize with many DNA-damaging chemotherapeutic drugs, and such synergy has been confirmed in a number of published studies. An important goal for further studies is to assess clinical efficacy in combination with relevant chemotherapeutic and targeted drugs in various tumor types. Ultimately, APR-246 may allow greatly improved therapy of a wide range of tumors that carry mutant TP53.

AUTHOR CONTRIBUTIONS

KW contributed to writing the manuscript and preparing the figures, communicated with the journal editor, and submitted the manuscript. VB, QZ, MZ, SC, and LA contributed to writing the manuscript and preparing the figures.

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Conflict of Interest Statement: Vladimir J. N. Bykov and Klas G. Wiman are co-founders and shareholders of Aprea AB, a company that develops p53-based cancer therapy, including APR-246. Klas G. Wiman is a member of its board. Lars Abrahmsen is employed by Aprea AB. 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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Clinical Overview of MDM2/X-Targeted Therapies

Andrew Burgess[1,2†], Kee Ming Chia[1†], Sue Haupt[3], David Thomas[1,2], Ygal Haupt[3] and Elgene Lim[1,2]*

[1] The Kinghorn Cancer Centre, Garvan Institute of Medical Research, Sydney, NSW, Australia, [2] Faculty of Medicine, St. Vincent's Clinical School, UNSW Australia, Sydney, NSW, Australia, [3] The Sir Peter MacCallum Department of Oncology, the University of Melbourne, Melbourne, VIC, Australia

MDM2 and MDMX are the primary negative regulators of p53, which under normal conditions maintain low intracellular levels of p53 by targeting it to the proteasome for rapid degradation and inhibiting its transcriptional activity. Both MDM2 and MDMX function as powerful oncogenes and are commonly over-expressed in some cancers, including sarcoma (~20%) and breast cancer (~15%). In contrast to tumors that are p53 mutant, whereby the current therapeutic strategy restores the normal active conformation of p53, MDM2 and MDMX represent logical therapeutic targets in cancer for increasing wild-type (WT) p53 expression and activities. Recent preclinical studies suggest that there may also be situations that MDM2/X inhibitors could be used in p53 mutant tumors. Since the discovery of nutlin-3a, the first in a class of small molecule MDM2 inhibitors that binds to the hydrophobic cleft in the N-terminus of MDM2, preventing its association with p53, there is now an extensive list of related compounds. In addition, a new class of stapled peptides that can target both MDM2 and MDMX have also been developed. Importantly, preclinical modeling, which has demonstrated effective *in vitro* and *in vivo* killing of WT p53 cancer cells, has now been translated into early clinical trials allowing better assessment of their biological effects and toxicities in patients. In this overview, we will review the current MDM2- and MDMX-targeted therapies in development, focusing particularly on compounds that have entered into early phase clinical trials. We will highlight the challenges pertaining to predictive biomarkers for and toxicities associated with these compounds, as well as identify potential combinatorial strategies to enhance its anti-cancer efficacy.

Keywords: p53, MDM2, MDMX, cancer therapy, nutlin

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****Correspondence:***
Elgene Lim
e.lim@garvan.org.au

†Andrew Burgess and Kee Ming Chia contributed equally to this work.

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INTRODUCTION: RATIONALE FOR TARGETING THE p53 PATHWAY

The tumor suppressor protein p53, nominated "the guardian of the genome," is mutated in ~50% of all human cancers. However, the incidence of p53 mutations differs significantly between cancer types, ranging from near universal mutation (~96%) in serous ovarian cancer to rare occurrence (<10%) in thyroid cancer (**Figure 1A**). This disparity provides therapeutic opportunities for targeting cancers with p53 wild-type (WT), in a distinct manner from those with p53 mutant cancers. Several preclinical studies have demonstrated that reconfiguration of mutant, to its normal,

active WT p53 conformation, restores apoptosis and promotes tumor regression (1–3). Therapeutic targeting of mutant p53, using small molecule drugs, is in the most advanced state for PRIMA-1, and its derivative PRIMA-1MET, an approach which restores the normal, active conformation of p53, which has been previously explored in depth by Wiman and coworkers (4). In the current review, we focus on therapies that target MDM2 and MDMX as a means of increase the stability of WT p53 and the consequences for patients with either WT p53 or mutant cancer cells.

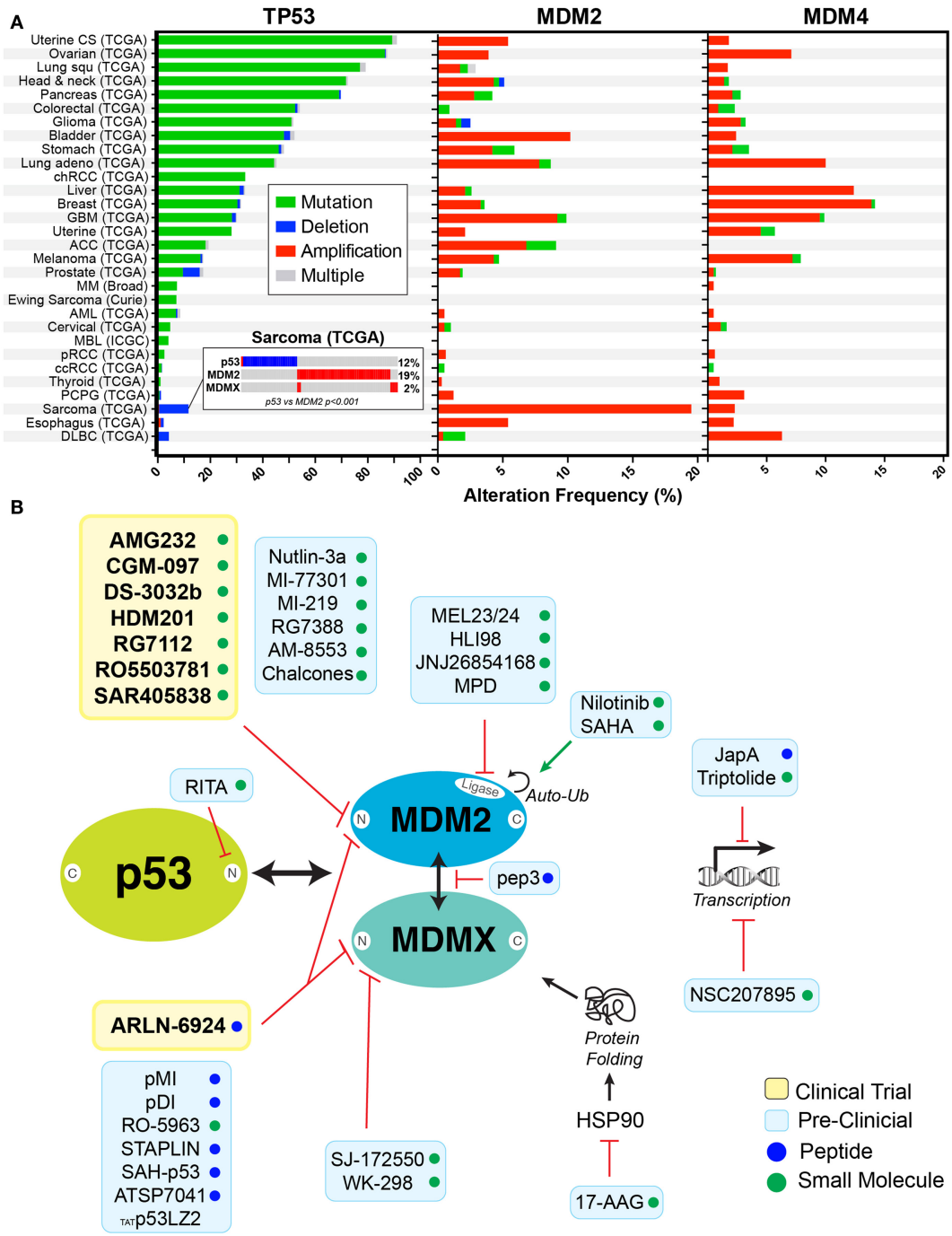

FIGURE 1 | Rationale for targeting p53 in cancers. (A) Frequency of alterations are shown with mutation (green), deletion (blue), amplification (red), and combination of alterations (gray) in p53, MDM2, and MDMX in cancers derived from cBioPortal (5) (http://www.cbioportal.org). Insert shows the mutual exclusivity observed between MDM2 expression and p53 deletion in sarcomas. **(B)** Schematic representation of inhibitors in clinical trials (yellow box) or in preclinical studies (blue box) targeting the p53–MDM2/X axis. Compounds are either small molecules (green circle) or peptide (blue circle).

Regulation of p53 Stability by MDM2 and MDMX

The primary response to a variety of cellular insults and stresses is to concurrently activate and stabilize p53 within the cell. Activated p53 then drives a vast transcriptional program that arrests the cell cycle, promotes repair pathways, and in response to severe stress initiates apoptosis. Therefore, under normal conditions, it is critical that intracellular levels of p53 are kept low, which is achieved by the rapid degradation of p53 by the proteasome. This degradation occurs in both ubiquitin-dependent (6) and ubiquitin-independent mechanisms (7) and can be modulated by various signaling pathways including sumoylation, phosphorylation, acetylation, methylation, and glycosylation (8). Of these, ubiquitination is the most important (6, 9) and the E3 ligase MDM2 is the primary negative regulator of p53 (10, 11), although several other E3 and E4 ligases of p53 also exist (8, 9). Mechanistically, engagement of the p53 N-terminal transactivation domain by the N-terminal of MDM2, facilitates its C-terminal RING finger E3 ligase activity to transfer ubiquitin to multiple lysine residues of p53, located in central DNA-binding and C-terminal regulatory regions (8, 9). MDM2 ubiquitination of p53 (either mono- or poly-ubiquitination) negatively regulates its transcriptional activity. Mono-ubiquitin triggers nuclear export, while poly-ubiquitin targets nuclear p53 for degradation by the proteasome (12). Notably, the C-terminal of MDM2 is also able to bind with the C-terminal of the highly related protein MDMX (also known as HDMX and MDM4). Although MDMX does not possess E3 ligase activity, the MDM2–MDMX heterodimer ubiquitinates p53 with higher efficiency than MDM2 homodimers (13). MDMX, via its N-terminus, is able to bind p53 and efficiently inhibit its transcriptional activity (14). Furthermore, MDM2 is transcriptionally up regulated by p53 and this negative-feedback loop associated with cyclical modulation of levels of both proteins, ensures that p53 levels remain low under normal conditions (15).

Targeting MDM2 and MDMX

Given the importance of both MDM2 and MDMX in regulating WT p53, it is unsurprising that they are commonly over-expressed in some cancers, including sarcoma (~20%) and breast (~15%) (**Figure 1A**). In this context, they function as powerful oncogenes and represent logical therapeutic targets for increasing WT p53 expression and activities. The concept of MDM2 targeting was supported by the discovery of $p14^{ARF}$ ($p19^{ARF}$ in mice), an alternate reading frame protein produced from the *CDKN2A* locus (16, 17). $P14^{ARF}$ binds to MDM2, sequestering it in the nucleolus and preventing it from targeting p53 for degradation (18, 19). More precisely, the capacity to bind and sequester MDM2 to the nucleus was assigned to a 22 amino acid fragment from the N-terminus of $p14^{ARF}$, revealing a potential method for targeting MDM2 with small peptide inhibitors (20). The first successful realization of this potential came in 2004, when nutlin-3a was discovered by Vassilev et al. (21). Nutlin-3a potently binds to the hydrophobic cleft in the N-terminus of MDM2, preventing its association with p53. Importantly, it is highly effective killing of WT p53 cancer cells, both *in vitro* and *in vivo* in preclinical models, provided validation for its use. However, its poor bioavailability, high toxicity (discussed in greater detail below), and its limited effects on MDMX overexpressing cells (22–24) has prevented its translation to the clinic. Recent interest has switched to compounds that have better bioavailability and can target both MDM2 and MDMX. These new compounds can be broadly segregated according to their mode of action. The vast majority of preclinical and clinical small molecule inhibitors work similarly to nutlin-3a, binding to the N-terminal pocket of MDM2, inhibiting association with p53 (**Figure 1B**). Despite the similarity in the N-terminal p53-binding domain of MDM2 and MDMX, most of these small molecule inhibitors bind with significantly less avidity to MDMX and are therefore primarily MDM2 specific (12). However, there are now several new peptide-based inhibitors that are capable of binding to the N-terminal of both MDM2 and MDMX (**Table 1**). In addition, several small molecule inhibitors, which bind specifically to the N-terminus of MDMX, have recently been developed and are currently undergoing preclinical testing (25, 26). In addition, there are now a growing number of new MDM2/X inhibitors that bind outside the N-terminus (**Figure 1B**). These include small molecules that inhibit the ubiquitin ligase activity of MDM2 (27); disruptors of MDM2–MDMX heterodimerization (28); transcriptional inhibitors of both *MDM2* (29, 30) and *MDMX* (31); MDM2 auto-ubiquitination activators (32, 33); inhibitors of HSP90 to disrupt MDMX protein folding; and molecules that directly engage p53 and prevent association with MDM2/X (34).

Cellular Responses to Increased p53

Increased cellular p53 protein levels, resulting from MDM2/X inhibition, lead to a number of effects that can be simplified into the broad categories of cell cycle arrest and apoptosis. The decision between these two pathways is governed by the level and duration of p53 induction. Lower and cyclical levels of p53 induce arrest, while sustained levels of elevated p53 expression promotes death (35). Cell cycle arrest is primarily achieved through transcriptional activation of p53 target genes, primarily p21 and GADD45, which block the activity of cyclin-dependent kinases (Cdk) and cause arrest in G1/S (36) and G2 phases, respectively (37). Interestingly, upregulation of p53 during mitosis does not delay mitotic progression, but it is an important requirement for arresting and eliminating aberrant polyploid cells in the subsequent G1 phase (38, 39). Continued p53 expression occurs when the damage or stress incurred cannot be repaired or resolved. These stresses continue to generate a signaling cascade (e.g., ATM/ATR, Chk1/2) that leads to the continued stabilization of p53, and subsequently allows the accumulation of pro-apoptotic p53 targets, including PUMA, Noxa, and Bim within the cell (40, 41). Once these proteins accumulate to sufficient levels, they trigger apoptosis (42, 43).

MDM2/X INHIBITORS IN CLINICAL TRIALS

The majority of MDM2-targeted therapies currently in clinical development are small molecule inhibitors (**Table 1**). These have been crystallographically resolved and comprise derivatives that bind to MDM2 by mimicking Phe19, Trp23, and Leu26,

TABLE 1 | MDM2 and MDMX inhibitors in clinical development.

MDM2 inhibitors in clinical development						
Class and specificity	**Nature of compound**	**Compound**	**Status**	**p53**	**NCT identifier**	**Company**
Small molecule MDM2 antagonists	Cis-imidazoline	RG7112	Phase I in advanced solid and hematological cancers, and liposarcoma (completed)	n/a	NCT00559533	
		RG7112 with cytarabine	Phase I in acute myelogenous leukemia (completed)	n/a	NCT01635296	
		RG7112 with doxorubicin	Phase I in soft tissue sarcoma (completed)	n/a	NCT01605526	Roche
		RO5503781	Phase I in advanced solid cancers (completed)	n/a	NCT01462175	
		RO5503781 with cytarabine	Phase I in acute myelogenous leukemia (active but not recruiting)	n/a	NCT01773408	
		RO5503781 with abiraierone	Phase I/II in advanced prostate cancer (recruiting)	n/a	CRUKE/12/032	
	Spiro-oxindole	SAR405838	Phase I in advanced solid cancers (active but not recruiting)	n/a	NCT01636479	Sanofi-Aventis
		SAR405838 with pimasertib	Phase I in advanced solid cancers (recruiting)	n/a	NCT01985191	
	Imidazothiazole	DS-3032b	Phase I in advanced solid cancers (recruiting)	n/a	NCT01877382	Daiichi Sankyo
	Dihydroisoquinolinone	CGM-097	Phase I in advanced solid tumors (recruiting)	wtp53	NCT01760525	
	n/a	HDM201	Phase I in advanced solid and hematological cancers (recruiting)	wtp53	NCT02143635	Novartis
		HDM201 with ribociclib	Phase Ib/II in liposarcoma (recruiting)	wtp53	NCT02343172	
	Piperidines	MK4828 with cytarabine	Phase I in acute myelogenous leukemia (terminated)	n/a	NCT01451437	Merck
	Piperidinone	AMG232	Phase I in advanced solid cancers and multiple myeloma (recruiting)	n/a	NCT01723020	Amgen
		AMG 232 with trametinib and dabrafenib	Phase Ib/IIa in metastatic melanoma (recruiting)	n/a	NCT02110355	
	Pyrrolidine	RG7388	Phase 1 in polycythemia vera and essential ihrombocythemia (recruiting)	n/a	NCT02407080	Pegasys
Stapled peptide MDM2/X inhibitor	Peptide	ALRN-6924	Phase I in advanced solid cancers (recruiting)	wtp53	NCT02264613	Aileron

Data extracted from http://www.clinicaltrials.gov, accessed 1st December 2015.

which are key residues engaged by p53. ALRN-6924 (Aileron Therapeutics) belongs to a different class of therapeutics, which are stapled peptides designed to disrupt p53 interaction with both MDM2 and MDMX. A number of these compounds are also being evaluated clinically in combination with cytotoxics (doxorubicin and cytarabine), and also molecular-targeted therapies, including ribociclib (CDK4/6 inhibitor), dabrafenib (BRAF inhibitor), trametinib, and pimasertinib (MEK1/2 inhibitors). A number of these trials have excluded patients with p53 mutant tumors; however, the majority have not defined a clear biomarker for selection criteria, in keeping with the primary end points of safety and tolerability. It is of interest that a number of these phase 1 trials have yet to be reported even though accrual was started over 3 years ago, which is unusually long in a phase 1 setting.

RG7112 is the most developed in this class of compounds, and preclinical studies demonstrate strong binding to MDM2, and effective apoptosis, particularly in MDM2-amplified tumors (44). One of first clinical trials reported was in patients with liposarcoma, a tumor characterized by a high proportion of MDM2 gene amplification and wild-type p53 (45). The primary end point in this small neoadjuvant study of 20 patients was to assess tumor biomarkers of p53 pathway activation and cell proliferation. The results demonstrated an increase in intratumoral p53, p21, and macrophage-inhibitory cytokine 1 (MIC1, a secreted protein product of p53) concentrations, an increase in MDM2 mRNA expression and a small decrease in Ki-67 positive cells in the treated compared to the pretreated samples. Clinically, the results were modest, with one partial response and stable disease in 70% of the cohort. Importantly, there were serious adverse events (grade 3 or 4) experienced by 40% of the patients, the majority of which were hematological in nature.

RG7112 has also been evaluated in a phase 1 trial of patients with relapsed/refractory leukemia, such as AML, ALL, CML, and CLL (46). The most common toxicities were gastrointestinal and hematological in nature, 22% of patients experiencing grade 3 and 4 febrile neutropenia. There was clinical activity, particularly in the AML cohort, whereby 5 out of 30 evaluable patients achieved either a complete or partial response, and another 9 patients had stable disease. These numbers suggest useful single agent clinical activity, given the refractory nature of their disease to other therapies. MDM2 inhibition resulted in p53 stabilization and transcriptional activation of p53 target genes. Interestingly, two patients who had p53 mutations (G266E and R181L) also responded to RG7112 in this trial. The G266E is a gain-of-function (GOF) p53 mutation that upregulates CXC-chemokine expression and enhances cell migration (47), while R181L is capable of inducing MDM2 and instigating a cell cycle arrest, but not apoptosis (48). Consequently, these mutants (G266E and R181L) may still be sensitive to MDM2/X inhibitors, and hence patients with these mutations may benefit from these inhibitors.

Assessing the effects that MDM2/X inhibitors in the context of the various GOF p53 mutants will be of significant importance, as MDM2/X inhibition has the potential to increase the levels of GOF p53 mutants. Several GOF mutants have been shown to increase cell proliferation, metabolism, invasion, and chemoresistance in cancer cells (49–53). Consequently, inhibition of MDM2/X could place selective pressure on cancer cells with GOF p53 mutations, driving the clonal evolution of more aggressive cancer cells and exacerbating tumor growth and metastasis in patients. Alternatively, a recent preclinical study demonstrated that the novel small molecule NSC59984 activates p73, resulting in an MDM2-dependent degradation of GOF p53 and subsequent inhibition of tumor growth (54). Other possible explanations for the varied patient response include multiple clones being present with the tumor (only some of which are mutant), a retention of one wild-type allele, certain p53 mutations may still have functional p53 activity (55). Taken together, it is clear that much more work needs to be done to clearly identify biomarkers to improve patient selection for clinical trials of MDM2/X inhibitors. Furthermore, understanding the heterogeneity of p53 expression and the specific mutations within a patient's tumor prior, during and post treatment will also be of considerable importance for determining the suitability of treatment with MDM2/X inhibitors.

The clinical effect of MDM2 inhibitors on p53 reactivation, range from cytostasis to apoptosis, and a combination strategy may be more efficacious in certain contexts. Preclincal modeling with nutlin-3a has demonstrated improved anti-cancer activity in combination with cytotoxic- and molecular-targeted therapies, in different tumor types (45); however, the toxicity profile of the combination partner is a critical determinant of the success of such an approach clinically. The high incidence of hematological toxicities in the clinical trials of RG7112 would suggest that therapies with an overlapping side effect profile would not be suitable as combination partners (45, 46). A number of clinical trials combining MDM2 inhibitors with cytotoxics have completed accrual but have yet to be reported (**Table 1**).

TOXICITIES

A concern of p53 reactivating therapies is its effect on normal cells. These include the stabilization of p53 resulting in increased apoptosis in these cells. This was reflected in the clinical trial of RG7112 in lipoma, whereby the most common toxicity was hematological in nature, with a reported 30% of patients experiencing grade 4 neutropenia, and 15% experiencing sometimes prolonged grade 4 thrombocytopenia (45, 46). Whether hematologic toxicity correlates with prior exposure to genotoxic therapies is not known. There are also reports of an increased incidence of p53 mutations following prolonged nutlin-3a exposure (56), and concerns about this effect on the development of new cancers (57). Other potential off-target effects on MDM2 inhibitors include the loss of its ability to ubiquitinate other proteins, such as the steroid hormone receptors [estrogen receptor (ER) and androgen receptor (AR)] and Rb, as well as interference with MDM2's role in DNA repair and modifying chromatin structure (58). The clinical relevance of these potential long-term toxicities have not been reported in the current early phase trials.

CONCLUSION/PERSPECTIVE

Protein–protein interactions, once considered to be a major hurdle to p53 therapeutic development, can now be targeted with a growing number of small molecule inhibitors and stapled peptides. The strategies to overcome this Achilles heel in many cancers are increasingly varied, and build upon an understanding of the crystallographic structure of p53 and its interactions with its major inhibitors. Most of the major pharmaceutical companies have one or more lead compounds targeting MDM2/X, and many of these have only recently progressed from preclinical development into early phase clinical trials.

The effect of MDM2/X-targeting therapies range from cytostasis to apoptosis, and combinatory approaches with other cytotoxic therapies or therapies that target other major oncogenic pathways are logical approaches, and may allow for lower and better tolerated doses of both drugs to be administered. For example, in p53 mutant tumors, protection of normal cells can be achieved by triggering p53-dependent cytostatic effects with short, pulsed exposure to MDM2 inhibitors. This cyclotherapy can reduce the toxic side effect of chemotherapy in these p53 mutant patients (59). Alternatively, recent preclinical evidence has demonstrated that inhibition of MDM2 with nutlin-3a prevents repair of DNA damage, providing synthetic lethality with genotoxic agents, such as cisplatin (60). Importantly, this effect was independent of p53 status and could provide a rational for examining MDM2 combination therapy in p53 mutant patients. Getting the therapeutic index right is critical in patients. It is not surprising that hematological toxicities have been the most commonly reported and dose-limiting toxicities in the trials reported so far (45, 46). Long-term follow-up is also critical to evaluate for the clinical relevance of the potential effects of an increase in p53 mutations and other off-target effects of this class of compounds.

The three major biomarkers that have been used to evaluate therapeutic responses to MDM2/X inhibitors are p53 status, MDM2, and MDMX levels. Interestingly, the over expression of MDM2, MDMX, or mutation of p53 are often mutually exclusive. For example, liposarcoma, which is one of the first tumors in which MDM2 inhibitors have been evaluated (45), shows highly significant tendency toward mutual exclusivity (p-value <0.001) between overexpression of MDM2 (19%) and p53 mutation (12%) (**Figure 1A**) (5). Other tumors with similar trends of exclusivity include glioblastoma multiforme, melanoma, bladder, lung andenocarcioma, prostate, and ER-positive breast cancers. These tumors present an obvious starting point for trialing MDM2/X inhibitors in patients. The high rate of MDM2 overexpression in prostate and ER-positive breast cancers, and the ability of MDM2 inhibitors to ubiquitinate steroid hormone receptors, has led to the evaluation of this class of drugs in combination with endocrine therapies (CRUKE/12/032). It has also been shown that estradiol modulates a subset of p53 and ER target genes that can predict the relapse-free survival of patients with ER-positive

breast cancer, and that p53 activation with nutlin in combination with fulvestrant, a selective ER degrader, led to a greater degree of apoptosis *in vitro* (61).

Given the risk of mutations in p53 driving resistance to MDM2/X inhibitors, additional biomarkers need to be identified to maximize the chances of clinical success. This is highlighted by evidence that p53 mutation status as currently measured clinically, may not be an accurate representation of functional p53 activity (46). In support, the recent discovery that MDM2 inhibitor sensitivity could be predicted by a panel of 13 p53 transcriptional target genes (62) was subsequently shown to be based on a significant number of miss-classified p53 mutant cell lines (63). Removal of these lines unfortunately abolished the predicative power of the gene signature. An alternative approach would be to select for tumors with MDM2 amplification given the mutual exclusivity of p53 mutations and MDM2 amplification (64). However, MDM2 and MDMX have different and cooperative inhibitory effects on p53 activity, and therefore inhibitors of one may not be as effective in the setting of raised levels of the other protein (23). Thus, these biomarkers, while logical in their choice, unless further improved upon, may potentially exclude patients who may benefit from these therapies.

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All authors contributed to the preparation and writing of the manuscript.

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