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Targeting the ubiquitin-mediated proteasome degradation of p53 for cancer therapy

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Abstract

Within the past decade, there has been a revolution in the types of drugs developed to treat cancer. Therapies that selectively target cancer-specific aberrations, such as kinase inhibitors, have made a dramatic impact on a subset of patients. In spite of these successes, there is still a dearth of treatment options for the vast majority of patients. Therefore, there is a need to design therapies with broader efficacy. The p53 tumor suppressor pathway is one of the most frequently altered in human cancers. However, about half of all cancers retain wild-type p53, yet through various mechanisms, the p53 pathway is otherwise inactivated. Targeting this pathway for reactivation truly represents the “holy grail” in cancer treatment. Most commonly, destabilization of p53 by various components of ubiquitin-proteasome system, notably the ubiquitin ligase MDM2 and its partner MDMX as well as various deubiquitinating enzymes (DUBs), render p53 inert and unresponsive to stress signals. Reinstating its function in cancer has been a long sought-after goal. Towards this end, a great deal of work has been devoted to the development of compounds that either interfere with the p53-MDM2 and p53-MDMX interactions, inhibit MDM2 E3 activity, or target individual DUBs. Here we review the current progress that has been made in the field, with a special emphasis on both MDM2 and DUB inhibitors. Developing inhibitors targeting the upstream of the p53 ubiquitination pathway will likely also be a valuable option.

Keywords

p53; MDM2; MDMX; ubiquitination; proteasome; cell cycle; apoptosis; deubiquitinating enzyme; ubiquitin ligase

Introduction

The tumor suppressor p53 is a sequence-specific transcription factor that regulates the expression of many target genes. The biological function of p53 is to induce cell cycle arrest, apoptosis, or senescence in response to diverse stress, thereby preventing cells from malignant transformation and tumorigenesis [1–4]. Twenty years ago, p53 was famously coined as “guardian of the genome”, referencing its ability to stall cell cycle progression in the face of DNA damage and to initiate either repair or death [5]. Since that time, we have learned that loss of p53 function is practically a universal feature of cancer. More than half of all tumors harbor mutations in the *p53* gene, while the other half retain wild-type p53, yet employ a number of mechanisms to circumvent its function [2, 4, 6, 7]. Such mechanisms

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include, but are not limited to, overexpression of its negative regulators MDM2 or MDMX and genetic loss or epigenetic silencing of its positive regulator, the tumor suppressor ARF [8–13]. Germ-line mutations in p53 are found in the familial Li-Fraumeni syndrome, which is characterized by early-onset cancers in diverse tissues [14, 15]. Genetic inactivation of p53 results in spontaneous tumors in mice [16, 17]. Transgenic mice expressing hot-spot gain-of-function p53 mutations also develop tumors in various tissues [18, 19]. Thus, p53 plays an essential role in protecting the organism from cancer.

Structurally, p53 possesses an N-terminal, bipartite acidic transactivation domain, which makes contacts with basal transcription factors and co-activators allowing for the initiation of transcriptional activation at target genes [20–22]. The central DNA-binding domain mediates sequence-specific binding to target gene promoters [23–25]. Of the thousands of *p53* missense mutations identified in human cancers, the vast majority are found within this central DNA-binding domain [7, 26], suggesting that the role as a transcription factor is essential for p53's tumor suppressive functions. While the spectrum of mutations have varying degrees of phenotypic effects, they generally fall into two classes: those that disrupt residues required for making contacts with DNA and those that alter protein conformation and folding, which preclude DNA binding or result in decreased stability. p53 also contains several unique domains. A tetramerization domain located in its C-terminus facilitates tetramer formation, allowing for optimal transcriptional activity [25]. A basic regulatory region at the far C-terminus is required for transcriptional activation of specific target genes by allowing for sequence-specific binding and linear diffusion across DNA [27–29]. It is also known to recruit co-factors, such as the acetyltransferase p300, which modifies p53-bound nucleosomes, resulting in a more open chromatin state [30]. Additionally, a proline-rich domain at the N-terminus regulates the stability and apoptotic function of p53 [31–34] and has recently been shown to be critical for p53 activation in response to DNA damage, but not oncogenic or oxidative, stress [35].

Given the detrimental effects of p53 activation, it is essential that p53 is held at low levels and dormant state under nonstressed conditions in normal cells. This is mainly achieved through its interaction with the ubiquitin E3 ligase MDM2, which is assisted by its partner protein MDMX. MDM2 mediates ubiquitination of p53 and targets it for proteasomal degradation. In response to stress, the MDM2-mediated p53 degradation is unleashed through various mechanisms, leading to p53 stabilization and activation. Given that half of human tumors retain wild-type yet functionally inactivated p53, largely due to deregulated suppression by MDM2 and/or MDMX, restoration of wild-type p53 function has become an attractive therapeutic goal. As proof-of-principle, several mouse models have shown that reactivating wild-type p53 function, after it has been impaired, results in tumor regression (see below). Within the past decade, numerous efforts have been undertaken to develop strategies aimed at reactivating p53. In this review, we will highlight current progress being made toward targeting p53 stability, specifically with regard to strategies that alter its ubiquitination status and prevent its proteasomal degradation. These include abrogating the MDM2-p53 interaction and inhibiting the enzymatic activity of the p53 ubiquitination pathway.

Biological Function of p53

Broadly, any extrinsic or intrinsic insult that threatens the integrity of the genome will elicit p53 activation. As a transcription factor, p53 activates or suppresses the expression of many protein-coding genes. These gene products mediate one of three major programs: cell-cycle arrest, apoptosis, or senescence. Additional functions include induction of autophagy, inhibition of angiogenesis, aid in DNA repair, and the regulation of metabolism [4]. The factors that determine which of these outcomes are chosen are still poorly understood.

However, the duration, extent and tissue in which the damage occurs likely influence this decision [4].

Upon activation, p53 binds to its cognate DNA response elements (REs) and directly regulates gene transcription. More than 4000 putative p53 REs are present within the genome [36]. While the vast majority of these sites have yet to be experimentally validated, attempts are being made to narrow the list by increasing the stringency of criteria required to define bona fide p53 targets. Using a more rigorous definition, ~129 high confidence targets have been identified and this list is growing [37]. Approaches such as global chromatin immunoprecipitation (ChIP) paired with expression data will increase our knowledge about the p53 network [37–39]. Although the picture is increasing in complexity, many canonical targets have been well characterized for each of the p53 output programs. For example, the p53 target gene, *CDKN1A* that codes for p21, is a cyclin dependent kinase (CDK) inhibitor that induces G1 cell cycle arrest. By inhibiting CDK mediated phosphorylation of Rb, p21 prevents the Rb-E2F complexes from dissociation and this precludes E2F from activating factors required for progression through the cell cycle [40, 41]. This downstream function protects genome integrity by allowing DNA damage repair and thwarting the buildup of damage-induced mutations. This was nicely illustrated by a p53 mutant (R172P) that is competent for inducing cell cycle arrest, yet defective in inducing apoptosis. Mice with a homozygous knockin of the R172P mutation show delayed onset of lymphoma and a high degree of chromosome stability in the resulting tumors. When R172P mice are crossed with *p21*^{-/-} mice the resulting sarcomas and lymphomas were aneuploid with numerous chromosome abnormalities, demonstrating that p21 acts as the primary p53 effector for maintaining genomic stability [42]. Although *p21*^{-/-} null mice are not predisposed to early onset tumor formation, this study suggests, that p21 defects, due to the resulting instability, may potentially select for more aggressive tumors. Other targets such as 14-3-3-σ and GADD45 have been implicated in G2/M phase arrest [43, 44]. If the damage persists or is too extensive to repair then the cell can enter a phase of irreversible cell-cycle arrest, known as senescence. This is mediated in part by p21, but other factors are clearly required to ensure its irreversibility. For example, it was shown that both constitutive signaling through the growth-regulating kinase, mTOR (mammalian target of rapamycin), and a prolonged duration of arrest cooperate with activated p21 to induce senescence [45, 46]. These senescent cells are then likely cleared by the immune system [47]. A second more direct option for eliminating permanently damaged cells is through the induction of apoptosis. Many pro-apoptotic p53 targets have been identified including those involved in the intrinsic apoptotic pathway, such as BAX [48], NOXA [49], PUMA [50], p53AIP1 [51] and BID [52].

p53 also regulates the expression of protein coding genes indirectly through the expression of a number of microRNAs (miRNAs). For example, p53 activates the expression of miR-34 family members (miR-34-a, -b, and -c). These members repress several target genes involved in cell cycle control or apoptosis, including cyclin E, CDK4, and BCL2 [53–55]. Similarly, p53 activates the transcription of miR-145, which suppresses the expression of c-Myc [56], an oncoprotein and a transcription factor essential for cell growth and proliferation. Also, p53-induced miR-107 targets hypoxia inducible factor-1β (HIF-1β) in the regulation of hypoxic signaling and anti-angiogenesis [57]. These miRNA-mediated effects are consistent with the tumor suppressive function of p53 (reviewed in [58]).

In addition, p53 also executes its function through protein-protein interactions. It has been shown that p53 interacts directly with several anti-apoptotic proteins such Bcl-XL and Bcl-2 to induce mitochondrial outer membrane permeabilization (MOMP), leading to cytochrome c release and apoptosis [59–61]. Finally, p53 also interacts with DNA repair pathway proteins to play a function in direct DNA repair [62–66].

Interestingly, a recent study indicates that p53 may also mediate tumor suppression through mechanisms other than cell-cycle arrest, apoptosis or senescence. Mice in which three critical acetylation sites (K117, K161, and K162) were replaced with arginines (p53^{3KR/3KR}) are unable to induce apoptosis, cell-cycle arrest or senescence following γ -irradiation. Surprisingly tumor progression was still delayed, in sharp contrast to the phenotype observed for p53 null mice [67]. Because p53^{3KR} retains the capacity to regulate metabolic targets, it is still effective at inhibiting glucose uptake, glycolysis and the accumulation of reactive oxygen species. Thus, this study implies that other biological function of p53, such as regulation of energy metabolism, may contribute to its tumor suppressive ability. Clearly, a thorough understanding of which p53-mediated outputs occur following its reactivation will be required to achieve the highest therapeutic efficacy.

Control of p53 stability through the ubiquitin-proteasome system: the p53-MDM2-MDMX axis

A considerable amount of cellular energy is invested in regulating the rates of protein turnover, which may vary depending on the target or context. The primary mechanism used to accomplish this is the ubiquitin-proteasome pathway. Although originally conceived of as an “intracellular garbage disposal” purposed to remove defective or misfolded proteins, it has now become clear that this system regulates virtually all aspects of cellular life [68]. Ubiquitin (Ub) is a highly conserved signaling molecule that when conjugated into polymeric chains (minimally four Ubs that are typically linked through the lysine 48 residue in Ub), serves to mark substrates for degradation through the 26S proteasome [69]. Adding to the complexity, various proteasome-independent ubiquitin functions, often associated with different lysine linkages, have also been identified. Ubiquitination is a highly ordered step-wise process that depends on three classes of enzymes: a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a ubiquitin ligase (E3). The first step of activation requires an E1 that utilizes ATP to catalyze the formation of ubiquitin adenylate. This charged intermediate then reacts to form a high-energy thioester bond with the catalytic cysteine in the E1. Ubiquitin is then transferred from the E1 to the active cysteine on an E2, again through the formation of a thioester bond. In the final step, the C-terminus of ubiquitin forms an isopeptide bond with an ϵ -amino group of a lysine residue either in the target protein or in another ubiquitin molecule, which is mediated by an E3 [70] (Fig. 1).

The process of ubiquitin conjugation is hierarchically organized, such that the number of enzymes encoded in the genome for each step successively increases. The human genome encodes two E1s (UBE1 and UBA6) [71, 72], at least 38 E2s, and more than 650 distinct E3s [73], each of which has the potential to recognize multiple substrates. Expression of E1s may vary based on tissue type such that each individual cell likely relies on a single E1 to charge all ubiquitin. Thus, E1s act on a global level and targeting this aspect of the pathway has less selective effects than downstream targets. All E2s share a ubiquitin conjugating (Ubc) domain of approximately 150 amino acids [74]. An E2 must interact with both an E1 and numerous E3s. Structural studies show that E1s and E3s share overlap in E2 binding interfaces, indicating that E1–E2 and E2–E3 interactions are most likely mutually exclusive [75]. A thorough understanding of the different E2–E3 complexes and the critical residues involved in binding will aid in the development of small molecules capable of targeting these interactions. The E3s represent the largest class of enzymes in the ubiquitin-proteasome system, with over 650 human E3s identified. This far exceeds the number of known human kinases (~500). Ubiquitin ligases have been classically split into two groups based on their general mode of catalysis. The HECT (homologous to E6-AP carboxy terminus) domain containing E3s have an identifiable catalytic site, whereas the RING (Really interesting new gene) domain-containing E3s are thought to mediate Ub transfer by

positioning E2s in close proximity to targets. RING proteins, and E3s in general, are regulated by a number of mechanisms that modulate either their activity or interactions [76].

As mentioned above, p53 is maintained at low levels, in an inert form, under physiological conditions, mainly owing to the negative regulation by MDM2. MDM2 binds to the transactivation domain of p53 through its N-terminal p53-binding domain, thereby directly inhibiting p53's transactivation activity [77, 78]. As an E3, MDM2 also mediates p53 ubiquitination and proteasomal degradation [79–82]. In addition, MDM2 promotes p53 nuclear export [83–85] and suppresses p53 acetylation by the crucial p53 coactivator p300 [86, 87]. Collectively, MDM2 suppresses p53 function and consistently, is overexpressed or amplified in many human cancers that retain wild-type p53 [10, 88–91]. As MDM2 is a p53 target gene that is upregulated following p53 activation, creating an elegant feedback loop [92–94]. This was verified by *in vivo* studies showing that a genetic knockout of p53 rescues the lethal phenotype of *mdm2* knockout mice [95, 96]. Furthermore, mice homozygous for a knock-in of an MDM2 E3-inactive mutant, C462A, are also embryonic lethal and can be rescued by deleting p53 as well [97]. Therefore, the E3 function of MDM2 is indispensable for its suppression of p53 *in vivo* [97]. p53 also induces a positive feedback loop by transactivating PIDD (p53-inducible protein with a death domain), which is a key component of the Caspase-2-PIDDosome. Upon formation of this complex, caspase 2 cleaves the N-terminal RING domain of MDM2, increasing p53 levels as well as cell survival, suggesting that cancer cells may take advantage of p53 in this case as a mechanism for increasing survival and drug resistance [98].

Another important negative regulator of p53 is MDMX, an MDM2 homolog and binding partner [99]. Like MDM2, MDMX also interacts with the transactivation domain of p53 through its highly conserved N-terminus and interferes with p53 transcriptional activity [100–102]. MDM2 forms either homodimers or heterodimers with its partner MDMX through the conserved function of their C-terminal RING domains [100, 103, 104]. Although MDMX contains a RING domain that is structurally similar to that of MDM2, it does not possess significant E3 activity [105, 106]. In spite of this, we know that MDMX is required for proper regulation of p53, both in the presence and absence of stress. Like MDM2, loss of MDMX, in mice, results in embryonic lethality that can be rescued by p53 deletion [107–109]. While it is not entirely clear how MDMX functions, several lines of evidence suggest that it modulates MDM2's activity. The MDM2 homodimer is relatively unstable due to its autoubiquitinating activity. When partnered with MDMX, on the other hand, the heterodimer is stable and selectively shifts ubiquitination from MDM2 towards p53 [102, 110]. Studies have also shown that MDMX may contribute to the E3 activity of MDM2 through residues in its extreme C-terminus. These residues, which are located outside of the RING domain of both MDM2 and MDMX, play a critical role in mediating E3 activity [110–112]. In MDM2, deletion of the C-terminus or point mutations that disrupt several aromatic residues render it incapable of ubiquitinating targets. For example, although it still interacts with p53 and is capable of forming homodimers, the MDM2 (Y489A) mutant does not possess E3 activity towards itself or p53, suggesting that Y489 is critical for that activity. Interestingly, heterodimerization of MDM2 (Y489A) with MDMX rescues E3 activity. This rescue is dependent on the extreme C-terminus of MDMX, indicating that the RING domain of MDM2 can cooperate with the extreme C-terminal amino acids in MDMX to affect E3 function [112]. MDMX has also been suggested to influence the site of ubiquitin conjugation, with a preference for C-terminal lysines in p53, whereas in its absence ubiquitin is directed to lysines within the DNA binding domain, at least *in vitro* [113]. This may be a result of MDMX's ability to induce conformational changes in p53 bringing different portions of the protein in contact with the E2 [113]. The C-terminus of MDMX may also recruit specific E2s that will determine the type of ubiquitin linkage and thus the fate of the substrate [114]. Supporting the essential role for MDM2-MDMX

heterodimerization in regulating p53 *in vivo*, mice with homozygous knockin of an MDMX point mutation (C462A) are embryonic lethal, which is also completely rescued by concomitant deletion of the *p53* gene [115]. Finally, adding another layer of complication to the matter, it was shown that adult mice with conditional deletion of the RING domain of MDMX (abolishing its interaction with MDM2) were viable and did not appear sick, suggesting MDM2/MDMX heterodimer is dispensable for regulating p53 beyond development [116]. This finding is interesting, as it would suggest that targeting MDMX for inhibition would be less toxic, offering a wider therapeutic window. Also, targeting MDMX could have other benefits, such as potential selection for MDM2 homodimer formation resulting in an overall decrease in MDM2.

p53 activation in response to stress

Numerous stress signals, including, but not limited to, DNA damage, hypoxia, telomere erosion, aberrant oncogene activation and ribosomal stress are known to induce p53, predominantly by inhibiting negative regulation imposed by MDM2/MDMX [117]. The result is increased p53 levels and its translocation to the nucleus, where it activates target gene expression. For example, DNA damaging agents induce and activate p53 primarily through the induction of a number of kinases, notably Ataxia telangiectasia mutated kinase (ATM), ataxia telangiectasia RAD3-related kinase (ATR), Chk1 and Chk2 which phosphorylate p53, MDM2, MDMX [118–125]. Early work suggested that a few critical sites may have been required for p53 activation following DNA damage. For example, phosphorylation at Ser 15, Ser 20 and Thr18 within the N-terminal transactivation domain decrease the interaction between p53 and MDM2 and subsequently promotes the recruitment of co-activators, such as p300 [126]. However, it now appears that these modifications are highly redundant as multiple kinases phosphorylate each site and further as demonstrated by knock-in mouse models, no single site appears to be critical for p53 regulation. Consistently, these are not sites commonly mutated in human cancers [127].

Aberrant oncogenic activation, such as overexpression of c-Myc, Ras, and E2F, etc., induces oncogenic stress, resulting in p53 stabilization and activation. This is mediated by the small basic nucleolar protein p14^{ARF} (p19^{ARF} in mouse) [128], another important tumor suppressor frequently deleted in human cancers. In response to aberrant proliferation and growth signals downstream of high oncogene activity, ARF binds to the central acidic domain of MDM2 and inhibits MDM2-mediated p53 ubiquitination and proteasomal degradation [129–131]. In addition, ARF also sequesters MDM2 into the nucleolus [132], although this effect is dispensable for its role in regulating p53 [133], and suppresses nuclear export of both MDM2 and p53 [130, 134].

Recently, it has been shown that ribosomal stress (also called nucleolar stress) can also activate p53 [135]. Ribosomal stress occurs following perturbation of ribosome biogenesis and can be induced in cells with a low dose of actinomycin D, 5-fluorouracil, mycophenolic acid, serum starvation, or knockdown of essential nucleolar components required for ribosome biogenesis [136–143]. An increasing number of studies including our own have shown that several ribosomal proteins (RPs), including L5, L11, L23, L26, S7, S27, S27a, S14, and S25 are molecular mediators responsible for p53 activation following ribosomal stress and this number is likely increasing [136, 138, 144–155]. These RPs interact with MDM2 and suppress MDM2-mediated p53 ubiquitination and degradation, leading to p53 stabilization, in response to ribosomal stress [135]. Clearly some RPs such as L5 and L11 are non-redundant MDM2 inhibitors, as knockdown of either L5 or L11 abolishes ribosomal stress-induced p53 activation by a broad range of treatments [136, 137, 140–142, 156, 157]. By contrast, other RPs such as L23 and S14 play a redundant role in regulating p53 following ribosomal stress, as knockdown of these RPs triggers p53 activation [138, 146,

154, 157]. Yet, whether RP-mediated p53 activation is due to the suppression of MDM2 E3 activity or enhancement of p53 deubiquitination is still open to debate. Also, why multiple ribosomal proteins regulate the MDM2-p53 loop is still not clear. Nevertheless, genetic knock-in of the L11- and L5- binding defective MDM2 mutant (MDM2^{C305F}) abolished ribosomal stress, but not DNA damage, induced p53 activation and significantly accelerated Eμ-Myc-induced lymphomagenesis in mice [158], firmly validating the essential role for L5 and L11 in mediating ribosomal stress-induced p53 activation *in vivo*. Interestingly, all above RPs bind to the central acidic and/or zinc finger domains of MDM2, suggesting that these centrally located regions contain critical regulatory domains that can be targeted for MDM2 inhibition.

Deubiquitination regulation of the p53-MDM2-MDMX pathway

Like other posttranslational modifications, ubiquitination is a reversible process. An entire family of proteins called deubiquitinating enzymes (DUBs) are dedicated to regulating this process. There are approximately 95 DUBs in the human genome that are grouped into five families based on homology within the catalytic domain: the ubiquitin-specific proteases (USPs), the ovarian tumor proteases (OTUs), the ubiquitin C-terminal hydrolases (UCHs), Machado–Joseph disease (MJD) protease, and JAB1/MPN/Mov34 metalloenzymes (JAMMs) [159, 160]. These DUBs are cysteine proteases relying on a canonical catalytic triad for their activity, with the exception the JAMMs, which are metalloenzymes. Recently, there has been a surge of interest in DUBs that regulate p53 signaling and a number of DUBs have been shown to regulate the p53-MDM2-MDMX pathway (Fig. 2). These DUBs are a mixture of both positive and negative regulators with varying degrees of specificity and different modes of regulation. A global examination of these DUBs reveals a complex yet elegant picture that enhances our understanding of p53 regulation.

The first and most well-characterized p53 DUB, USP7 (also known as HAUSP for Herpes-Associated Ubiquitin Specific Protease), was identified as a p53 binding protein by an affinity purification method [161]. Both *in vivo* and *in vitro* assays demonstrated that USP7 is a bona fide p53 DUB. Overexpression of USP7 induces p53 activation, leading to growth suppression and apoptosis. DNA damage increases the interaction between p53 and USP7 [161]. This landmark paper, which surfaced just a few years after we had begun to understand the relationship between p53 polyubiquitination and MDM2, set the field into motion. It was not long after this discovery that MDM2 was also recognized as a target of USP7 and suggested to be the true physiological substrate. This was hypothesized based on the fact that complete knockdown or loss of USP7 resulted in dramatic MDM2-dependent p53 activation, although consistently with its deubiquitinating activity towards p53, partial knockdown increased p53 degradation [162, 163]. This is pertinent when considering USP7 as therapeutic target because inhibitors that do not completely abolish its catalytic activity may have undesired effects. Structural studies have shown that p53 and MDM2 interact with the N-terminus of USP7 in a mutually exclusive manner [164]. Further, *in vitro* assays have shown that MDM2 may act as a bridge so that deubiquitination of p53 by USP7 can occur in the absence of a direct interaction between the two proteins [164]. Finally, with the addition of MDMX to the story, it became partially clear how this complex network operates. In the absence of DNA damage, USP7 preferentially stabilizes both MDM2 and MDMX and MDM2 and MDMX require USP7 for stability. Upon DNA damage the interaction between MDM2/MDMX and USP7 decreases in an ATM dependent manner [163]. Concomitantly, the interaction between USP7 and p53 increases. Overall, this body of work emphasizes that USP7 regulates p53 signaling in a context dependent manner and that inhibitors targeting this DUB may be quite effective when combined with DNA damaging agents.

USP10 was recently identified as the first p53 specific DUB. It deubiquitinates p53, but not MDM2 and MDMX [165]. It normally localizes to the cytoplasm and is thought to help maintain steady state levels of cytoplasmic p53. p53 ubiquitination and nuclear export are thought to be differentially regulated by MDM2 in a dose-dependent manner such that low levels of MDM2 induce monoubiquitination and nuclear export of p53 whereas high level of MDM2 activity mediated its polyubiquitination and nuclear degradation [166]. Under non-stressed conditions, USP10's activity toward p53 is relatively weak, probably due to its overall instability. Following DNA damage, ATM phosphorylates USP10, resulting in stabilization of USP10 and allowing it to more efficiently deubiquitinate and recycle cytoplasmic p53 back to the nucleus [165]. Also, a small portion of USP10 translocates to the nucleus to aid in p53 stabilization, presumably alongside USP7. Consistently, USP10 is significantly down-regulated in renal clear cell carcinomas [165], suggesting that USP10 may have tumor suppressive functions.

In contrast, USP2a differs from USP7 and USP10 in that it deubiquitinates MDM2 and MDMX, but not p53, leading to destabilization of p53 [167, 168]. Thus, USP2a has suspected oncogenic roles and is overexpressed in a subset of prostate cancers [169]. In this context USP2a regulates invasiveness by destabilizing p53 and interferes with its ability to transactivate microRNAs that negatively regulate c-Myc [170]. Aside from its known role in cancer, USP2a is also an attractive target because the identification of small molecule DUB inhibitors has been far more successful than the discovery or design of DUB agonists.

A second DUB that negatively regulates p53 signaling is USP4. In contrast to USP2a, USP4 deubiquitinates and stabilizes a different E3 that ubiquitinates p53 called ARF-BP1, thereby destabilizing p53 [171]. Although MDM2 is the predominant E3 regulating p53, others including COP1, ARF-BP1, and Pirh2 have recently been identified [172]. Given the potentially redundant activities of MDM2 and ARF-BP1, it will be interesting to examine mechanisms of resistance in MDM2 targeted therapies and determine whether ARF-BP1 is sufficient to reverse MDM2 inhibition in cancer. If so, targeting USP4 may be of considerable importance as a second line of therapy.

Of note, different DUBs may regulate p53 in response to different types of stress. USP29 is transcriptionally activated following oxidative stress and then mediates p53 deubiquitination. Overexpression of USP29 stabilizes p53 and induces p53-dependent apoptosis in HCT116 cells [173]. USP29 may be an important factor in resolving issues surrounding p53 outputs in response to oxidative stress and therefore a potentially useful biomarker [174]. On the other hand, functional outcomes of p53 activation are known to vary based on the strength, duration and type of stress encountered. Slight alterations in p53 levels at any point following the induction of stress may have a dramatic impact on the response elicited. Interestingly, USP42 appears to regulate p53 levels only during an early phase of the stress response and does not affect the basal levels of p53 in unstressed cells [175]. Although required for only a short window of time, USP42 is essential for a durable cell cycle arrest following treatment with several cytotoxic agents. By potentiating sustained arrest, USP42 stabilization of p53 may provide cells with the opportunity to repair damage, thereby likely promoting genome stability in normal cells [175]. It is interesting to determine whether USP42 will play a similar survival role in cancer and whether it could be a therapeutic target in cancer.

Aside from the above-mentioned USP family members, we recently identified the OTU family member DUB, Otubain 1 (Otub1), as a novel p53-positive regulator [176]. In contrast to the above USPs, Otub1 regulates p53 stability and activity primarily through a novel non-canonical mode that is independent of its DUB activity: suppression of the MDM2-cognate E2, UbcH5 (Fig. 2). Consequently, overexpression of Otub1 induces p53-dependent

apoptosis and inhibition of cell proliferation whereas knockdown of Otub1 attenuates p53 activation following DNA damage [176]. Consistently, Otub1 interacts with UbcH5. Thus, instead of targeting Otub1 directly, developing small molecule inhibitors to suppress UbcH5 activity based on the mechanism of Otub1 suppression could be another interesting strategy to reactivate p53. Recent structural studies of the E2-Otub1 complex [177–179] would likely promote structure-based design of such E2 inhibitors.

Mouse Models: rationale for p53 reactivation in cancer

Initially, it was unclear as to whether p53 defects were initiating events or whether they were required to sustain tumor growth. Several mouse models have elegantly shown that not only is inactivation of p53 required for tumor maintenance but reestablishing wild-type function can reverse tumor progression and increase overall survival. Interestingly, the models reveal that the p53 programs induced upon reactivation may vary depending of tumor type or stress. For example, mice that were irradiated in the absence of functional p53 developed both sarcomas and lymphomas. Interestingly, p53 reactivation in sarcomas or liver cancer led to senescence, whereas in lymphomas, apoptosis was observed [47, 180, 181]. These pioneering studies provided strong rationale for the development of p53-targeted therapies. However, one critique of these earlier studies was that p53 was being reactivated under conditions of excessive oncogene activation. This is important because low oncogenic activity is sufficient to drive tumorigenesis but does not necessarily result increased p53 signaling via ARF [182, 183]. Therefore, two recent studies revisited p53 restoration in context of lower oncogene signaling. Indeed both groups concluded that restoring p53 had no impact on low grade, early stage, adenoma-like lung cancers but dramatically reduced high grade adenocarcinoma subtypes [184, 185]. Further, upon restoration, the expression profiles of remaining tumors appeared to cluster with adenoma subtype. These studies have very broad implications for the use of p53 reactivating therapies in the clinic. First, this strategy will probably be most effective in late stage cancers versus early stage cancers, in which oncogene expression is lower, yet, future studies should evaluate this question in other tumor types. Second, at least in lung cancer, p53 reactivation may select for an adenoma-like subtype and therefore combination therapies with this in mind may succeed. Finally, timing may be extremely important factor when administering combination therapies that involve p53 reactivation.

Using a similar strategy, MDMX was recently evaluated for its efficacy as a target when p53 is subsequently reactivated. Based on the observation that MDMX degradation is required for complete p53 induction following DNA damage, it was hypothesized that loss of MDMX would further increase the p53 levels, perhaps surpassing thresholds enough to induce killing, even in cells with low oncogenic signaling [186, 187]. Indeed, Garcia et. al report that in the absence of MDMX, mice in which p53 is reactivated have an overall increase in survival [187].

Targeting the p53 ubiquitination pathway for cancer therapy

After more than 30 years of effort by the p53 community, we have gained a detailed understanding of p53 regulation, particularly molecular insights into the MDM2-p53 interaction and the multi-layered regulation of p53 by ubiquitination. Over past decade, many of these mechanistic and molecular revelations have been translated into strategies to drug p53. The first p53 product Gendicine, a recombinant adenovirus encoding human p53 approved in China in 2003, has been used locally for treating several types of solid tumors [188, 189]. Also, several small molecule inhibitors to reactivate mutant and wild-type p53 are either in pre-clinical phases or currently being tested in clinical trials (Table 1). In this

section, we will focus on the current progress made toward drugging p53 by targeting the ubiquitination system.

Targeting the MDM2-p53 interaction

The high-resolution crystal structure of the N-terminus of MDM2 complexed with a short p53 peptide has provided molecular details about the MDM2-p53 interaction. MDM2 contains a well-defined and relatively deep hydrophobic pocket in its N-terminus (residues 25–109) where the transactivation domain of p53 binds [190]. The minimal MDM2-binding site on p53 was mapped to residues 18–26 [191–193]. The MDM2 pocket is filled by the side chains of three key hydrophobic residues (Phe 19, Trp 23, and Leu 26) in the amphipathic helical region of the p53 peptide [190, 191]. This well-defined interaction has provided the basis for the design of small-molecule inhibitors that target this interaction.

The first of such inhibitors were the cis-imidazoline analogs, the nutlins [194], which have fueled enthusiasm for identifying other small molecule antagonists of MDM2. Nutlins bind to the hydrophobic pocket of MDM2 in a manner similar to p53 binding, thereby interfering with the interaction between MDM2 and p53. Nutlins, at lower micromolar concentrations, can induce nongenotoxic activation of p53, leading to cell cycle arrest and/or apoptosis in many cancer cell lines [194, 195] and primary leukemia samples ([196–198] and reviewed in [199]) in a wild-type p53-dependent manner. Nutlin-3, an analog of the nutlins, has potent *in vivo* activity against p53 wild-type human tumor xenografts in mice without causing significant cytotoxic effects [194, 195]. Consistently, its therapeutic index appears high, as it has been shown that nutlin-3 induces both cell cycle arrest and apoptosis in cancer cells, yet only induces cell cycle arrest in normal primary cells [194, 195]. Interestingly, the apoptotic response to Nutlin-3 treatment varies in different cancer cell lines [195]. Cancer cells with MDM2 amplification are more sensitive to undergoing apoptosis by nutlin-3 [195, 196, 200], whereas high levels of MDMX confer less sensitivity to nutlin-3-induced apoptosis [201–204]. Expression of other oncogenes may also sensitize cancer cells to nutlin-3. For example, neuroblastoma cells with MYCN amplification are more sensitive to nutlin-3-mediated and p53-dependent apoptosis and cell growth inhibition [205]. In addition, nutlin-3 also induces transcription-independent apoptosis in cancer cell lines possibly by binding to Bcl-XL [206] or promoting p53-Bcl-2 interactions [207], and thereby neutralizing the inhibitory effect of Bcl-2 on Bax/Bak. A recent phase I study in hematological malignancies have shown that nutlin-3 is well-tolerated with continuing dose escalation and stabilization and activation of p53 were evident [208].

Spiro-oxindole-derived compounds were identified through structural design to suppress MDM2 binding to p53 [209, 210]. The initial compound MI-63 binds to MDM2 with high affinity and induces p53 expression and p53-dependent cancer cell growth inhibition. However, this compound is not suitable for *in vivo* evaluation due to poor pharmacodynamics. Extensive optimization of this compound has led to a new derivative, MI-219, with better pharmacodynamics and bioavailability [211]. MI-219 binds to MDM2 with a *K_i* value of 5 nM and is highly selective for MDM2 over MDMX. It disrupts the MDM2-p53 interaction by binding to the MDM2 hydrophobic pocket and activates p53 pathway, leading to cell cycle arrest and apoptosis, which is selective for cancer cells at nanomolar concentrations. *In vivo* human tumor xenograft studies revealed that MI-219 has potent p53-dependent anti-tumor effect without severe cytotoxic side effects [211]. Also, *ex vivo* treatment with MI-219 induced p53-dependent apoptosis in primary leukemia samples [212, 213].

Recently, structure-based rationale design has led to the discovery of a novel and completely different class of compounds. Extensive medicinal chemistry optimization led to a compound named AM-8553, which is a potent and selective inhibitor of the MDM2-p53

interaction with excellent pharmacokinetic properties and *in vivo* efficacy [214]. The co-crystal structure of AM-8553 with MDM2 confirmed that the compound successfully mimics the three key p53 hydrophobic binding residues. Also, it forms an additional charge-charge interaction with His 96 of MDM2 [214]. AM-8553 treatment activates p53 in cells and *in vivo* and suppresses cancer cell proliferation in a p53-dependent manner. It is also effective in suppression of tumor growth in human xenograft tumors in mice. Although studies are needed to further determine its anti-tumor effect and pharmacokinetics, AM-8553 could have potential for further clinical development as another MDM2 inhibitor.

Another small molecule called RITA (reactivation of p53 and induction of tumor cell apoptosis) directly targets p53. Unlike the nutlins, MI-219 and AM-8553, RITA binds to p53 to disrupt the MDM2-p53 interaction, leading to p53 accumulation and apoptosis in various cancer cells retaining wild-type p53 and substantial p53-dependent anti-tumor effect *in vivo* [215]. It induces apoptosis most effectively in the context of oncogene expression, thereby sparing normal cells. Interestingly, RITA is more efficient at inducing apoptosis than nutlins [216] possibly due to its inhibition of additional cell survival factors [217]. Recent studies showed that RITA-induced apoptosis requires CHK2 activity [218] and that RITA also induces p53-dependent replication stalling through CHK1 [219], suggesting that the mechanism of RITA-induced apoptosis and cell growth inhibition is quite complex.

Finally, using a thermal denaturation assay to screen chemical libraries, Gresberger et al identified a novel series of benzodiazepinedione MDM2 antagonists, which disrupt the MDM2-p53 interaction [220]. Administration of the benzodiazepine derivative TDP665759 results in increased p21 levels in the liver of nude mice and synergizes with doxorubicin to induce apoptosis in cultured cancer cells and to inhibit human xenograft tumor growth in mice [221].

Targeting the MDMX-p53 interaction

MDMX partners with MDM2 to efficiently regulate p53 ubiquitination and degradation [222]. Thus for complete reactivation of p53 in cancer cells, it is also critical to target MDMX [223]. Besides, the efficacy of MDM2 inhibitors can be compromised by overexpression of MDMX, potentially providing a resistance mechanism [201–204]. This is primarily because current small molecule inhibitors targeting MDM2, such as nutlin-3 and MI-219, have a much lower affinity for MDMX than for MDM2 [195, 211, 224]. Designing inhibitors that block p53 interactions with both MDM2 and MDMX has proven difficult due to the differences in p53 binding sites. Recent structural studies [225–227] have revealed specific distinctions between the p53-binding cleft of MDMX and that of MDM2. For example, the Tyr 99 residue of MDMX is oriented differently when compared to the corresponding Tyr 100 residue of MDM2 and the bulkier Met 55 of MDMX corresponds to the Leu 54 residue in MDM2 [226, 227]. The total sum of these structural differences brings Y99 into close proximity with the larger M55 and this results in partial obstruction of the p53-binding cleft of MDMX. Consistently, the affinity of p53 binding to MDM2 is much higher than the affinity of its binding to MDMX. These structural studies make rationally designed inhibitors, specific for MDMX, feasible. Recently, the first MDMX inhibitor, SJ-172550, was identified in a high throughput screen [228]. This compound binds reversibly to the p53-binding pocket of MDMX, thus displacing p53. Consequently, in several MDMX-overexpressing cell lines, treatment with this inhibitor induced p53-dependent apoptosis. However, it seems that this compound did not induce significant accumulation of p53 in cells [228]. A later study showed that SJ-172550 forms a covalent albeit reversible bond with MDMX. This changes the protein conformation and renders it more susceptible to reducing agents, suggesting a rather complex multi-modal mechanism of inhibition and perhaps limiting the compound's value as a lead MDMX inhibitor [229]. Thus, there is an emerging need for developing more potent and specific MDMX small

molecule inhibitors. WK298 (or Novartis-101), optimized from the imidazo-indo scaffold series, inhibits p53-MDMX binding with single μM K_i potency (see references in [230]). This promising compound may have potential for development as a dual MDM2/MDMX inhibitor.

Several alternative strategies for targeting MDMX are currently also under development. These include peptide inhibitors as well as small molecules that suppress MDMX expression. Importantly, MDMX overexpression in cancer cells is primarily caused by aberrant transcription or gene amplification [231]. Therefore targeting this aspect of its regulation in the correct context should be highly effective. Recently, a benzofuroxan derivative called XI-006 (NSC207895) was identified as an MDMX inhibitor [232]. Instead of targeting the p53-MDMX interaction, this compound inhibits MDMX transcription in various cancer cells, although the mechanism of action is unclear. Consequently, XI-006 treatment induces the levels and activity of p53 via down-regulating MDMX, leading to p53-dependent apoptosis in cancer cells. Additionally, combining XI-006 with nutlin-3 showed additive effects in cancer cells. Recently developed peptide inhibitors show some efficacy in inhibiting MDMX, however still appearing to bind MDM2 more efficiently. Examples include PMI (TSFAEYWNLLSP) [233] and a recently described stapled p53 helix [234].

Targeting the ubiquitin E3 activity of MDM2

Another approach to reactivating p53 is to directly suppress MDM2 ubiquitin ligase activity. Indeed, Yang et al [235] identified a family of small molecules closely related to the 7-nitro-5-deazaflavin compounds named HLI98s as inhibitors of the MDM2 E3 activity in a high throughput screen. These compounds inhibit MDM2-mediated p53 ubiquitination and induce p53-dependent apoptosis in cancer cells [235]. In MEFs, these compounds did not stabilize p53 in the absence of MDM2, indicating that they do not inhibit other E3s that target p53 for degradation. The major drawback for this class of compounds is their lack of solubility in aqueous solution and their relatively low potency. Also, HLI98 treatment results in p53-independent cell-cycle arrest and apoptosis, indicating off-target activities of these compounds. Further medicinal chemistry study showed that the nitro group in the initial HLI98 compounds is actually not essential for 5-deazaflavin to activate p53 [236]. Recently, a highly soluble and more potent HLI98 homolog called HLI373 was discovered [237]. This compound inhibits MDM2-mediated p53 ubiquitination at low micromolar concentrations and has greater potency than the HLI98s in stabilizing p53 and activating p53-dependent transcription as well as in inducing p53-dependent apoptosis in several cancer cell lines. As expected, these active 5-deazaflavin compounds inhibit MDM2 E3 activity by specifically binding to the RING domain of MDM2 [238]. Future studies will determine whether this binding interferes with MDM2's homodimerization or dimerization with MDMX as well as its interaction with E2s. Another MDM2 E3 inhibitor called sempervirine was recently uncovered from high throughput screening of natural products [239]. Like 5-deazaflavin analogs, sempervirine also suppresses MDM2-mediated p53 ubiquitination, stabilizes p53, and induces apoptosis in wild-type p53 containing cancer cells [239]. These studies establish that small molecule compounds inhibiting the E3 activity of MDM2 may have potential as novel cancer therapeutics.

Targeting DUBs to reactivate p53

DUBs have emerged as important regulators of cancer pathways. Various DUBs display different tolerances for different residues in their active-site clefts [240]. Thus, it appears possible to design small molecules that selectively target the active sites of different DUBs. Indeed, several specific DUB inhibitors have recently been identified, including inhibitors against the viral DUB PLPro [241, 242], the human proteasome-associated DUB USP14

[243], and USP1 implicated in the Fanconi anemia pathway [244]. Likewise, great attempts are being made to identify inhibitors of MDM2 specific DUBs in an effort to reactivate p53 in cancer cells.

Since MDM2 is the preferred target of USP7 under unstressed conditions [162, 163], then logically, suppression of USP7 should destabilize MDM2. Several small molecule inhibitors that target USP7 have recently been identified with such effects. Using high throughput screening, Colland et al [245] initially identified a cyano-indenopyrazine derivative small molecule compound HBX 41,108 as a USP7 inhibitor. This compound stabilizes p53, inhibits cell proliferation, and induces p53-dependent apoptosis in cancer cells. It was later found that HBX 41,108 also inhibits several other tested DUBs such as UCHL3, USP5, USP10, and USP8 [246]. In contrast, two other small molecule compounds, HBX 19,818 and HBX 28,258, were identified as USP7-specific inhibitors working at low micromolar concentrations [246]. This specificity is conferred by the ability of these compounds to form covalent binding to the catalytic cysteine (Cys223) of USP7 [246]. Similar to the cellular effects of RNAi-mediated knockdown of USP7, these compounds destabilize MDM2 and induce p53, leading to G1 cell cycle arrest, apoptosis, and cell growth inhibition in cancer cells, providing proof-of-concept that a USP7 inhibitor could be a valuable therapeutic option in cancer. Whether these compounds inhibit cancer cell proliferation independent of p53, for example through other USP7 targets such as FOXO4, PTEN, Claspin [247], remains to be determined. Another screen for USP7 inhibitors and subsequent medicinal chemistry optimization identified P22077 as a novel small molecule inhibitor of USP7 (and the closely related USP47) [248]. Treatment with this compound led to a drastic accumulation of polyubiquitinated proteins in cells, indicating that USP7 may target a broad range of substrates. Consistently, treatment with P22077 reduced MDM2 and induced p53 in cancer cells. Whether this compound inhibits cancer cell growth *in vivo* awaits further investigation.

A recently discovered small molecule called Spautin-1, developed as a potent inhibitor of autophagy, was shown to target USP10 and USP13 [249]. Given the role of USP10 as a positive regulator of p53 [165], its use, as an anti-cancer therapeutic, will likely depend on p53 status. Spautin-1 may have high efficacy in a context where mutant p53 plays an oncogenic role.

Recently, it has been shown that chalcone-based small molecule inhibitors AM146, RA-9 and RA-14 suppress cancer cell proliferation via targeting several DUBs including UCH-L1, UCH-L3, USP2, USP5 and USP8 [250]. In addition to down regulating positive cell cycle regulators such as cyclin D1, these compounds also induce p53. This is consistent with the known role of USP2 as a negative regulator of p53, through its deubiquitination and stabilization of MDM2 and MDMX [167, 168]. A specific USP2 inhibitor would be more desirable for further cancer therapeutics.

Targeting E2s to reactivate p53

Currently, approaches to reactivate p53 through the inhibition of E2s are not available. However, targeting E2s to suppress cancer cell proliferation has recently been reported. Ceccarelli et al [251] identified the first small molecule inhibitor called CC0051, which selectively inhibits the human CDC34, an E2 enzyme critical for the proteasomal degradation of cell-cycle regulatory proteins by functioning with the cullin-RING ligase (CRL)-based E3s [252]. Intriguingly, CC0651 does not target the catalytic site of hCDC34. Instead, it inserts into a cryptic binding pocket on hCDC34 away from the catalytic site, causing allosteric conformational change of hCDC34 [251]. Consequently, CC0651 specifically interferes with the ubiquitin transfer to substrates, without affecting hCDC34's interactions with E1 or E3 enzymes or the formation of the ubiquitin thioester. Consistently,

CC0651 analogs caused an accumulation of the SCF^{Skp2} substrate p27 in cells and inhibited proliferation of human cancer cell lines in a CDC34-dependent manner [251]. Although CC0651 is not acting as an active site inhibitor, this finding is exciting and sets the stage for the discovery of new E2 inhibitors. Noncatalytic site inhibition of E2 activity may represent a viable class of drug targets that selectively inhibit an E2 enzyme. Identifying inhibitors of the MDM2 cognate E2s, such as UbcH5 to reactivate wild-type p53 would be extremely interesting.

Targeting E1 to reactivate p53

Yang Y et al [253] has identified a cell-permeable pyrazone compound called PYR-41 that inhibits the E1 activity. This compound induces the levels of p53 and preferentially induces apoptosis in transformed cells with wild-type p53. As an E1 inhibitor, it is not surprising that PYR-41 also induces other proteins involved in the immune response and may be applicable as an anti-inflammation agent. The advantages of inhibiting the ubiquitin proteasome system at different points along the pathway remain unknown. For example, whether targeting a single E3 has an advantage over targeting an E2 that has multiple substrates still need further examination. In spite of this, an inhibitor towards an E1 was recently developed by Millenium Pharmaceuticals has shown selectivity in inducing cell cycle arrest and apoptosis in cancer cells [254]. One unanswered question is to what extent p53 stabilization and activation plays a role in mediating E1 inhibitor-induced cellular effects.

Targeting the proteasome

Finally, bortezomib is the first and only drug that targets the ubiquitin-proteasome system that has been approved for clinic use, due to its success in treating a number of cancers. It covalently and reversibly binds to the 20S catalytic core and inhibits proteasome activity. Bortezomib has been approved for treating multiple myeloma, B cell lymphoma, and mantle cell lymphoma and is in advanced stage clinical trials for many other tumor types (reviewed in [255–257]). It is known that many defects involved in the development and progression of cancer occur through the ubiquitin-proteasome system. Thus, non-specific inhibition of protein degradation could have advantages in terms of targeting different defected pathways in different cancer types. For example, bortezomib was shown to stabilize the inhibitory molecule I κ B leading to the suppression of the activity of NF- κ B, which is frequently altered in multiple myeloma (reviewed in [257, 258]). Among the many other targets, p53 is undoubtedly stabilized by bortezomib. Whereas a number of studies showed that p53 is required for induction of apoptosis induced by proteasomal inhibitors [260–263 and references in 258], other studies also reported that bortezomib can induce apoptosis independent of p53 [264–267]. This seemingly conflicting data highlights the importance of understanding the underlying biological context. Nevertheless, the clinical success of bortezomib is a true inspiration to our continuing effort in targeting the p53 ubiquitination system in cancer. Furthermore, there are a number of proteasome inhibitors currently in clinic trials [255]. On the other hand, a small molecule called b-AP15 was recently discovered as a 19S proteasome inhibitor [259], by specifically suppressing the deubiquitinating activity of the two proteasome-associated DUB, UCHL5 and USP14, in the 19S proteasome. Treatment with b-AP15 suppressed tumor growth in several xenograft human tumor models in mice. Again, these effects were not dependent on the status of p53 [255]. It is interesting to evaluate whether proteasome inhibitors have a more dramatic therapeutic effects in cancers that retain wild-type p53.

Conclusions and perspectives

The past decade has seen the success of drug development through targeting phosphorylation pathways. With our increasing understanding of the catalytic mechanisms

and protein interactions operating within the ubiquitin proteasome system, rationally targeting this system has begun to emerge as another key goal in drug design. Of the numerous pathways regulated by the ubiquitin proteasome system, p53 is one of the most important and thus extensively studied pathway due to its central position as a regulatory hub that is frequently altered in human cancer. Recent discoveries of several small molecule compounds that reactivate p53 have generated a great deal of enthusiasm about their potential and broader applications in cancer. Some of the compounds are in pre-clinical studies, while others are in early stage clinical trials. There is, however, still much to be done through combined efforts of cancer biologists, medicinal chemists, physicians, and pharmaceutical enterprises. Continued dissection of both molecular and biochemical mechanisms operating within the p53 regulatory pathway is still the fulcrum for new drug discovery.

Looking into the future, new approaches targeting the p53 ubiquitination pathway remain vital. Since MDM2 heterodimerizes with MDMX to mediate p53 polyubiquitination and degradation [115, 222], screening small compounds that inhibit the MDM2-MDMX interaction may be an alternative option to suppressing MDM2's activity towards p53. Biochemical and crystal structure studies have shown that residues at the C-terminal ends of both MDM2 and MDMX forming a β strand are essential for their heterodimerization [110–112]. The MDM2-MDMX heterodimer or their homodimer interactions involve the contacts of the β strand at the C-terminal end of one molecule and a β strand at the middle of the ring-finger domain of the other molecule. Together with a third β strand in the middle of the ring-finger domain of each molecule, the interactions form a six β strand barrel [110]. Thus, it is likely that this barrel structure could be disrupted by small molecules or peptides that bind to the two key β strands involved in the interaction. The central region of MDM2 contains the acidic and zinc finger domains critically implicated in the regulation of p53 ubiquitination and have not been explored for drug design so far. Many proteins that regulate MDM2 E3 activity, including the tumor suppressor ARF and MDM2-binding RPs, bind to these regions. While the mechanism by which these proteins regulate the E3 activity of MDM2 remains unclear, small molecules or peptides that target this central region will likely emerge in the near future, although this region is unstructured. One possibility is to design highly basic small molecules such as stapled peptides [268] that mimic ARF or RP binding to MDM2 to suppress MDM2 activity. Alternatively, directly manipulating RPs, through the selective inhibition of RNA polymerase I with compounds such as CX-5461 has already shown efficacy [269]. As noted in the main text, targeting MDM2 cognate E2s to reactivate p53 is currently not available, however the success of CC0651 which inhibits another E2, CDC34, has shown promise in killing cancer cells [251]. Because individual E2s contact multiple E3s to ubiquitinate different substrates, targeting MDM2's cognate E2s would nonspecifically affect other substrates as well. However, given that the proteasome inhibitor, bortezomib, affects multiple pathways yet shows significant efficacy in treating cancers, this broader specificity may actually prove advantageous. In addition, the E2–E3 interactions are relative weak [73]. Thus, targeting the E2-MDM2 interaction to abrogate the p53 ubiquitination would be another, maybe easier, approach. Finally, the rational combination of therapies based on patient biomarkers will result in the largest therapeutic window. In sum, although questions remain, novel clinical therapeutics, targeted at the reactivation of p53, are not only highly desirable but also expected options in cancer treatment.

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Figure 1.

A Schematic diagram showing the p53 ubiquitination and degradation pathway through the ubiquitin-proteasome system. Numbers indicate where strategies have developed to target the pathway to reactivate p53.

Figure 2.

Regulation of the p53-MDM2-MDMX pathway by DUBs. p53 is ubiquitinated by MDM2, which is assisted by MDMX. MDM2 also mediates ubiquitination of MDMX and itself. In addition, ARF-BP1 ubiquitin ligase also mediates p53 ubiquitination. A number of DUBs have been shown to regulate this pathway (see text for details).

Table I

Small molecule compounds targeting the p53 pathway for reactivation

Compound	Targets	Trials	References
Nutlin-3	MDM2-p53 interaction	Phase I	[194]
Spiro-oxindoles (MI-63, MI-219)	MDM2-p53 interaction	Progress to phase I	[209–211]
AM-8553	MDM2-p53 interaction	Cells,	[214]
RITA	MDM2-p53 interaction	Cells, mouse	[215]
Benzodiazepine (TDP665759)	MDM2-p53 interaction	Cells, mouse	[220,221]
SJ-172550	MDMX-p53 interaction	Cells	[228]
XI-006	MDMX transcription	Cells	[232]
HLI98s/HLI373	MDM2 E3	Cells	[235, 237]
Sempervine	MDM2 E3	Cells	[239]
PYR-41	E1	Cells	[253]
UAEi	E1	Cells	[254]
HBX 41108	USP7	Cells	[245]
HBX 19818 HBX 28258	USP7	Cells	[246]
P22077 (P5091)	USP7	Cells	[248]
Spautin-1	USP10 and USP13	Cells	[249]
CCX-651	RNA Pol I inhibitor	Cells, mouse	[269]
Bortezomib	20S proteasome	Approved	[255–258]
b-AP15	19S proteasome (USP14 and UCHL5)	Cells, mouse	[259]