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REVIEW

Deubiquitinating enzyme regulation of the p53 pathway: A lesson from Otub1

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Abstract

Deubiquitination has emerged as an important mechanism of p53 regulation. A number of deubiquitinating enzymes (DUBs) from the ubiquitin-specific protease family have been shown to regulate the p53-MDM2-MDMX networks. We recently reported that Otub1, a DUB from the OTU-domain containing protease family, is a novel p53 regulator. Interestingly, Otub1 abrogates p53 ubiquitination and stabilizes and activates p53 in cells independently of its deubiquitinating enzyme activity. Instead, it does so by inhibiting the MDM2 cognate ubiquitin-conjugating enzyme (E2) UbcH5. Otub1 also regulates other biological signaling through this non-canonical mechanism, suppression of E2, including the inhibition of DNA-damage-induced chromatin ubiquitination. Thus, Otub1 evolves as a unique DUB that mainly suppresses E2 to regulate substrates. Here we review the current progress made towards the understanding of the complex regulation of the p53 tumor suppressor pathway by DUBs, the biological function of Otub1 including its positive regulation of p53, and the mechanistic insights into how Otub1 suppresses E2.

Key words: p53; MDM2; Ubiquitination; Deubiquitinating enzymes; Otub1; Cell cycle; Apoptosis

Core tip: p53 is tightly regulated by dynamic ubiquitination and deubiquitination. A number of deubiquitinating enzymes (DUBs) have been shown to regulate p53 stability and activity by either directly deubiquitinating p53 or indirectly deubiquitinating its regulators. We recently discovered that Otub1, an OTU family DUB, stabilizes and activates p53 *via* distinct and non-canonical mechanism wherein it suppresses the MDM2 cognate ubiquitin-conjugating enzymes UbcH5. Here we review the current progress made towards the understanding of the Otub1 functions as a potent E2 inhibitor and the underlying mechanisms.

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MDM2 AND MDMX: KEEPING P53 UNDER CONTROL

The p53 tumor suppressor plays a central role in maintaining the genomic stability and preventing the organism from cancer[1-3]. Loss of p53 function, either through direct mutations in the *p53* gene or indirectly through alterations in the p53 regulatory networks, is associated with most, if not all, human cancers[4,5]. Germline mutations of *p53* result in the cancer-prone Li-Fraumeni

syndrome in human[6] and deletion of the *p53* gene leads to spontaneous tumors in mice[7,8]. p53 is a stress-induced transcription factor that activates or represses the expression of many target genes, thereby executing its anti-proliferative activity by inducing cell cycle arrest, apoptosis, or senescence[1,2,9-11]. Under normal circumstances, p53 is tightly controlled at low levels mainly by its negative regulator MDM2[12-14]. As a RING-finger-containing ubiquitin ligase (E3)[15,16] MDM2 mediates p53 ubiquitination and degradation through the proteasomal system[17,18]. MDM2 also directly suppresses p53 transactivation activity by binding and concealing the N-terminal transactivation domain of p53[19-21]. The centrality of the MDM2-mediated p53 suppression has been demonstrated by mouse genetic studies showing that deletion of the *mdm2* gene caused embryonic lethal phenotype, which is completely rescued by concomitant deletion of *p53*[22,23]. This essential function of MDM2 requires its E3 activity, as mice with homozygous knock-in of the E3 inactivation mutant, $MDM2^{C464A}$, are also embryonic lethal, which can be rescued by deleting p53 as well[24]. Consistently, MDM2 is overexpressed in a number of human cancers, most of which contain wild-type p53[25-29].

The MDM2 homolog MDMX has emerged as an equally important p53 regulator as MDM2[30]. MDMX shares high homology with MDM2 in their C-terminal RING-finger domain and the N-terminal p53-binding domain. Like MDM2, MDMX binds to the N-terminal transactivation domain of p53 and suppresses its activity. However, MDMX does not have appreciable ubiquitin ligase activity towards p53[31,32], yet it assists MDM2 to suppress p53 function. MDMX directly binds to MDM2 *via* their RING domains[33-35] and renders MDM2 sufficiently stable to ubiquitinate and degrade p53[33,36-38]. Also, MDMX suppresses p53 function by specifically promoting p53-induced MDM2 transcription following DNA damage[39]. MDM2, in turn, ubiquitinates and degrades MDMX in response to DNA damage[40-42]. Thus, the mutual regulation between MDM2 and MDMX ensures a proper cellular level and activity of p53. Supporting the indispensible role of MDMX towards p53, deleting the *p53* gene also rescues the lethal phenotype of knocking out the *mdmx* gene in mice[43-45]. Like MDM2, MDMX is also overexpressed or amplified in several types of human cancers that harbor wild-type p53[46-49]. Recent studies have provided further molecular insights into the non-redundant and indispensible role for MDMX in MDM2-mediated p53 degradation. First, like MDM2, the RING domain of MDMX and resulting MDM2-MDMX heterodimerization are required for the regulation of MDM2, as deletion of the RING-finger domain of MDMX or knock-in of the MDM2-binding defective MDMX mutant (C462A) resulted in embryonic lethal phenotype, which was completely rescued by deletion of *p53*[50,51]. Second, The extreme C-terminal short sequences outside of the RING domain of both MDM2 and MDMX contribute to the MDM2 E3 activity, owing to their role in the formation of MDM2-MDMX heterodimer and perhaps the E3 holoenzyme mediating p53 polyubiquitination[37,38,52]. Third, a recent *in vitro* study has shown that while MDM2 alone is sufficient to mediate multi-monoubiquitination of p53, the MDM2-MDMX complex is required for p53 polyubiquitination[53]. Thus, the stoichiometry of the p53-MDM2-MDMX complex is critical for the determination of whether targeting p53 for polyubiquitination or monoubiquitination.

The p53-MDM2-MDMX axis is among the most highly regulated pathways. Enormous molecules regulate the interplay among the three proteins in response to diverse stressors, leading to p53 stabilization and consequent activation. These include various post-translational modifications of all three proteins. Ubiquitination plays a key role in controlling the protein stability and activity of all three proteins. Under stress conditions, p53 ubiquitination mediated by MDM2/MDMX is crippled as a result of either dissociation of MDM2/MDMX from p53 or suppression of MDM2/MDMX activity towards p53. For example, DNA damage-mediated phosphorylation of both p53 and MDM2 disrupts their interaction, resulting in p53 stabilization[54-57]. DNA damage also triggers phosphorylation and degradation of MDMX, alleviating its suppressive effect on p53[58-63]. Oncogenic stress induces p53 *via* suppression of MDM2 by ARF[64-68], whereas ribosomal stress induces p53 *via* suppression of MDM2 by a number of ribosomal proteins[69-85]. Again, ARF also promotes MDM2-mediated MDMX degradation[40] and ribosomal stress-induced p53 activation requires MDM2-mediated MDMX degradation[86]. Thus, barricading the inhibition of p53 imposed by MDM2 and MDMX is centrally important for p53 activation in response to most, if not all, stressors. Indeed, both MDM2 and MDMX bind to p53 at its target gene promoters and suppress its transactivation activity[87-89]. Thus, p53 activation is thought to involve the release of such repression, called anti-repression under stress conditions, through diverse posttranslational modifications[90]. In addition, p53 is also ubiquitinated by a number of other ubiquitin ligases such as ARF-BP1[91], PIRH2[92], COP1[93], *etc.*[94,95]. For example, p53, under certain cellular levels, is thought no longer regulated by the MDM2/MDMX complex. Instead, the basal level of p53 is mainly regulated by ARF-BP1. Deletion of ARF-BP1 completely activates p53 in the presence of MDM2[91]. Adding to the complexity of the ubiquitination regulation of the p53 pathway, deubiquitination regulation has recently emerged as an equally important mechanism for p53 control.

REGULATION OF THE P53-MDM2-MDMX PATHWAY BY DEUBIQUITINATING ENZYMES

Like other posttranslational modifications, ubiquitination of p53, MDM2 and MDMX can be reversed through a process called deubiquitination, which is catalyzed by a different class of enzymes called deubiquitinating enzymes (DUBs). The human genome encodes approximately 95 predicted DUBs that are classified into

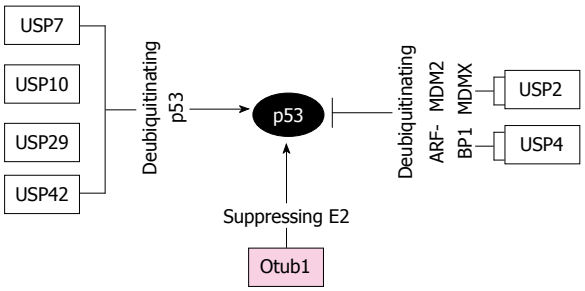

Figure 1 Diagram of the regulation of the p53 pathway by deubiquitinating enzymes. Arrows indicate activation and bars indicate inhibition. USP7, USP10, USP29, and USP42 deubiquitinate and activate p53, whereas USP2 destabilizes p53 by deubiquitinating MDM2 and MDMX and USP4 destabilizes p53 by deubiquitinating and stabilizing ARF-BP1. Otub1 stabilizes and activates p53 *via* non-canonical suppression of the MDM2 cognate E2 UbcH5, thereby inhibiting MDM2-mediated p53 ubiquitination and degradation. USP: Ubiquitin-specific protease

5 families: ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), ovarian tumor associated proteases (OTUs), Machado-Joseph disease (or Josephin domain) proteins (MJDs), and JAB1/MPN/MOV34 proteins (JAMMs). Except that the JAMMs are zinc metalloproteases, all other DUBs are cysteine proteases[96,97].

Recently, several DUBs from the USP family have been shown to regulate the p53-MDM2-MDMX loop (Figure 1). USP7, also called herpesvirus associated USP (HAUSP), is the first DUB reported to be a bona fide p53 deubiquitinase[98-100]. Overexpression of USP7 stabilizes and activates p53[99]. Intriguingly, MDM2 seems to be a better substrate of USP7 compared to p53 under physiological circumstances, as substantial knockdown of USP7 results in destabilization of MDM2 and activation of p53[98,101]. Further, USP7 also deubiquitinates MDMX in cells and *in vitro* and depletion of USP7 results in destabilization of the otherwise stable MDMX[100]. DNA damage triggers ATM-dependent phosphorylation of MDMX, which disrupts its binding to USP7 and leads to the consequent increase of ubiquitination and degradation of MDMX[100], whereas the interaction between p53 and USP7 is increased following DNA damage. Thus USP7 scrutinizes the homeostatic levels of p53, MDM2, and MDMX under both normal and stress conditions. The second p53 DUB, USP10, has also been shown to play a critical role in p53 activation following DNA damage[102]. Unlike USP7, USP10 is a cytoplasmic DUB and specifically deubiquitinates p53, but not MDM2 and MDMX[102], reversing MDM2-mediated ubiquitination, nuclear export, and cytoplasmic degradation of p53. Following DNA damage, ATM phosphorylates USP10 at Thr42 and Ser337, resulting in not only the stabilization of USP10, but also the translocation of a fraction of USP10 into the nucleus to deubiquitinate and activate p53. Consistent with its function in regulating p53, USP10 expression is down-regulated in high percentage of clear cell carcinomas[102]. Recently, USP42 was reported to be another DUB that positively regulates p53 stability and activity. Interestingly, USP42 deubiquitinates p53 only during the early stages of stress response, without significant effect on p53 regulation under unstressed conditions. Despite of this, it has been shown that USP42 is required for rapid p53 activation and cell cycle arrest in response to mild or transient DNA damage stress[103]. In addition, Liu *et al*[104] has shown that USP29 positively regulates p53 stability and function following oxidative stress. This is achieved by the increased transcription of USP29 induced by oxidative stress, which in turn cleaves polyubiquitinated p53, leading to p53-dependent apoptosis in cells.

In contrast to above USPs positively regulating p53, USP2a and USP4 were reported to destabilize p53 and suppress p53 function, albeit *via* targeting different p53 E3s. USP2a destabilizes p53 by deubiquitinating and stabilizing both MDM2[105] and MDMX[106], whereas USP4 destabilizes p53 by deubiquitinating and stabilizing ARF-BP1[107]. Consistently, USP2a is overexpressed in a subset of prostate cancers[108,109], whereas USP4 is overexpressed in a broad range of human cancers[107]. Thus, USP2a and USP4 are likely oncogenic DUBs.

Together, these studies demonstrate that deubiquitination plays a crucial role in finely tuning the normal homeostasis of the p53-MDM2-MDMX loop as well as its response to stress. They also imply that different DUBs could regulate the p53 pathway *via* different mechanisms within different cellular compartments following different stress. However, whether p53 is regulated by DUBs other than USP family members is previously unknown. We recently identified that the OTU domain-containing ubiquitin aldehyde-binding proteins 1 (Otubain 1, Otub1 thereafter), an OTU family DUB, controls p53 stability and activity *via* a novel non-canonical mechanism[110].

OTUB1: A UNIQUE MEMBER OF OTU DUB FAMILY

Otub1 was identified along with its close homolog Otub2 by affinity purification using the DUB-specific inhibitor, Ub aldehyde[111]. Subsequent studies, including our own, revealed that Otub1 possesses *in vitro* deubiquitinating enzyme activity preferentially towards K48-linked polyubiquitin chains[110,112,113]. Like other cysteine proteases, Otub1 contains a catalytic triad consisting of Cys (C) 91, His (H) 265, and Asp (D) 268[112]. However, crystal structure studies demonstrated that Otub1 possesses unique structure features wherein H265 is located distantly from the catalytic C91 and D268 and the access of C91 to ubiquitin is blocked by Glu (E) 214 residue, forming a conformation incompatible with catalysis by typical cysteine proteases[112], implying that the activity of Otub1 may be highly regulated in cells and its activation may be subjected to conformational change (See below). Otub1 is ubiquitously expressed in tested human tissues. A longer isoform called Otub1 ARF (alternative reading frame)-1, resulting from alternative splicing and start codon, is predominantly expressed in peripheral blood mononuclear cells, lymph nodes, spleen, and the tonsils[114]. The function of Otub1 ARF-1 is thought to antagonize the function of Otub1 in cells[114].

Functionally, Otub1 has been implicated in the regula-

tion of immune response, estrogen signaling, DNA damage response, as well as pathogen biology. Soares *et al*[114] first reported that Otub1 regulates $CD4^+$ T cell clonal anergy by enhancing degradation of the ubiquitin ligase called GRAIL (gene related to anergy in lymphocytes) and promoting interleukin 2 production following antigenic stimulation, whereas the Otub1 ARF-1 has an opposite effect. Interestingly, the effect of Otub1 does not depend on its catalytic activity. As a matter of fact, the role of Otub1 in degrading GRAIL is opposite to its predicted role as a DUB[114]. A possible explanation is that Otub1 forms a ternary complex with GRAIL and USP8, another USP family DUB, thereby suppressing the deubiquitination of GRAIL by USP8. In this case, Otub1 may act as an ubiquitin editing protease[114]. Li *et al*[115] reported that Otub1 (and Otub2) mediate virus-induced deubiquitination of TNF receptor-associated factor 3 (TRAF3) and TRAF6, two ubiquitin ligases required for virus-induced Interferon regulatory factor 3 (IRF3) and NF-kB activation, leading to the inhibition of viral-induced production of INFβ. However, whether this effect requires the DUB enzymatic activity of Otub1 is not clear[115]. Further, Otub1 has recently been shown to enhance TGFβ signaling by inhibiting ubiquitination and degradation of SMAD2/3[116]. Otub1 also plays a role in pathogen invasion of the host cells. The *Yersinia*-encoded virulence factor YpkA interacts with and phosphorylates Otub1[117] and recruits the small GTPase RhoA, leading to the stabilization of the active RhoA[118]. Consequently, overexpression of wild-type, but not the C91S mutant, Otub1 increased the susceptibility of host cells to the *Yersinia* evasion[118]. Otub1 has been shown to deubiquitinate and stabilize ERα in chromatin[119], albeit this stabilization results in the inhibition of ERα-mediated transcription. Adding to the complexity, the catalytic mutant Otub1, C91S in which the catalytic C91 is mutated to S, did not abolish Otub1-mediated suppression of ERα activity[119]. Otub1 has been shown to inhibit DNA-damage-induced chromatin ubiquitination, which is also independent of its DUB activity. Instead, Otub1 suppresses RNF168-dependent chromatin polyubiquitination by binding to and inhibiting the RNF168 cognate E2 enzyme UBC13[120]. Recently, Otub1 has been shown to regulate apoptosis by deubiquitinating the cellular inhibitor of apoptosis (c-IAP1)[121].

Together, Otub1 has been implicated in multiple biological processes. In most cases, the effects of Otub1 do not require its DUB activity, such as the regulation of DNA damage-induced chromatin ubiquitination[120], T-cell anergy[114], ERα[119], and SMAD2/3[116], implying a unique model of ubiquitination regulation by a DUB: suppression of the ubiquitin-conjugating enzyme (E2) (see below). Because of this and the fact that it is expressed in most tissues, Otub1 may have a broad function in cells.

OTUB1 IS A NOVEL POSITIVE P53 REGULATOR

We recently found that Otub1 positively regulates the stability and activity of p53[110]. Overexpression of Otub1, but not its close homolog Otub2, markedly stabilizes and activates p53 and induces p53-dependent apoptosis and cell growth inhibition. Interestingly, Otub1 regulation of p53 does not require its catalytic activity, as mutating C91 to either A or S did not abolish the activity of Otub1 to block MDM2-mediated p53 ubiquitination and degradation, to stabilize and activate p53, and to induce p53-dependent cell growth inhibition[110]. Mechanistically, Otub1 suppresses MDM2-mediated p53 ubiquitination by binding to and inhibiting the MDM2 cognate E2 enzyme UbcH5s[110]. This is consistent with the non-canonical role for Otub1 in suppressing DNA damage-induced chromatin ubiquitination by inhibiting UBC13[120]. Therefore, our study further supports that the suppression of substrate ubiquitination through inhibiting cognate E2s by Otub1 represents a unique noncanonical mode of DUB regulation compared to classical cysteine proteases and this may be a general mechanism for Otub1 to regulate the substrate protein ubiquitination and stability.

Consistent with the noncanonical mode of regulation, mutating C91 to either A or S did not abolish the activity of Otub1 to bind to and suppress UbcH5[110]. However, a point mutation of Asp 88 to Ala ($Otub1^{D88A}$) abolished the function of Otub1 to suppress p53 ubiquitination and degradation and this mutant interacts with p53 stronger than wild-type Otub1, indicating this mutation might create a dominant-negative effect. D88 is located closely to the donor ubiquitin-binding surface and thus its mutation would affect the binding of Otub1 to donor ubiquitin conjugated to UbcH5. Although D88 is not located directly in the E2 binding surface, our experimental data revealed that this mutation clearly disrupted the Otub1-E2 interaction in cells[110]. This might be due to the overall structure change after D88 mutation. Supporting this conformational change is that D88A mutant also results in the loss of Otub1's DUB activity.

Our functional studies of the endogenous Otub1 suggest that Otub1 plays an important role in p53 stabilization and activation following DNA damage induced by diverse agents. This is consistent, but not completely, with the observation that Otub1 suppresses DNA damage-induced chromatin ubiquitination, thereby suppressing DNA repair pathway[120]. One explanation is that upon DNA damage, Otub1 might target UbcH5-MDM2 to stabilize p53, while it may dissociate from the RNF168-Ubc13 complex, allowing RNF168 to catalyze K63-linked chromatin ubiquitination and subsequent DNA repair response. Whether DNA damage-induced posttranslational modification plays a role in this functional switch remains unclear. However, phosphorylation of Otub1 has been observed at several residues such as T134. Further, it has been shown that the phosphorylation mimicking Otub1 mutant T134E, but not T134A, failed to rescue the DNA damage response in Otub1-depleted cells[122]. Thus it is interesting to examine the signaling pathways involved in the phosphorylation of Otub1 and how this phosphorylation plays a role in regulating Otub1 function in

response to DNA damage stress.

MECHANISTIC INSIGHTS INTO THE NON-CANONICAL SUPPRESSION OF E2 BY OTUB1

Recent biochemical and structural studies have shed a light on how Otub1 suppresses E2s[122-124]. It has been shown that Otub1 preferentially binds to ubiquitin-charged E2[120,122]. Otub1 contains two ubiquitin-binding motifs: a distal site that binds to free ubiquitin and a proximal site that binds to donor ubiquitin conjugated to the active site of an E2 (*e.g.,* Ubc13 or UbcH5). The structure of two ubiquitin binding to Otub1 is reminiscent of that of K48-linked di-ubiquitin[122]. Interestingly, the binding of a free ubiquitin to the distal site allosterically causes the conformational change of Otub1, allowing the formation of a N-terminal ubiquitin-binding helix where the E2-charged donor ubiquitin then binds[122,124]. Consequently, this binding limits the donor ubiquitin interaction with the backside of another E2 and the attack on the thioester bond by an acceptor ubiquitin, a step important for ubiquitin transfer[122,124]. On the other hand, Otub1 also makes contacts with E2 and the Otub1-binding surface in E2 (UbcH5 and Ubc13) overlaps with the E3-binding surface. Thus this Otub1-E2 interaction may also attenuate the E2-E3 engagement[122,124]. Collectively, Otub1 is a potential inhibitor of the E2 enzymes. Further supporting this notion, Otub1 has recently been shown to be a major DUB that interacts with the D and E classes of E2 as well as UbcE2N[125]. Thus disruption of the Otub1-E2 interaction or donor ubiquitin-Otub1 interaction would theoretically abolish Otub1's activity to suppress E2. This could distinguish Otub1's E2 suppressing activity from its DUB enzyme activity. Indeed, several mutants involved in the E2-contacting surface of the Otub1, such as F133A, T134R, F138A, have been shown to lack the E2-suppressing activity but retain the DUB activity[122,124]. Therefore, it is interesting to examine whether these mutants could fail to stabilize and activate p53 in cells. On another note, we recently found that Otub1 can be monoubiquitinated by UbcH5 and this monoubiquitination in turn plays a critical role in the Otub1's E2 suppressing activity. We further found that UbcH5 preferentially binds to monoubiquitinated Otub1, through the ubiquitin interaction with the backside ubiquitin-interacting surface of E2[126]. This binding could potentially disrupt the formation of self-assembled ubiquitin-charged UbcH5 (UbcH5-Ub) conjugates that is critical for ubiquitin transfer, polyubiquitin chain formation and efficient polyubiquitination of substrates[127,128], suggesting another novel mechanism of Otub1 suppression of E2.

CONCLUSION

Recent studies have convincingly demonstrated Otub1 as a unique DUB that executes diverse biology functions by non-canonically suppressing E2 enzymes. Therefore it is expected that Otub1 may play broad functions in cells. One question would be how these broad functions coordinate with each other in cells. We also do not know how Otub1's activity is regulated in cells. Interestingly, a recent observation showed that Otub1 DUB activity can be regulated by UbcH5, which stimulates the binding of the Lys48-linked polyubiquitin substrate by stabilizing the folding of the N-terminal ubiquitin-binding helix of Otub1, thereby promoting its deubiquitinating enzyme activity[129]. It is interesting to know how these mutually regulatory functions are controlled in cells. It is also important to test how Otub1's activity and levels are regulated in cells under physiologic and stress conditions. As Otub1 is a potent activator of p53[110] and plays a role in DNA damage repair[120], Otub1 may act as a tumor suppressor. Thus it is important to determine whether Otub1 is deregulated in human cancers. Gene targeting in mice could provide further information regarding the function of Otub1 and whether Otub1 indeed possesses tumor suppression function *in vivo*. Further characterization of mechanistic insights into the Otub1 suppression of E2 could also be useful for developing strategies that target the E2 enzymes for cancer therapy, *e.g.,* small molecule compounds that resemble Otub1 interaction with E2.

Together, p53 is ubiquitinated by MDM2/MDMX and several other E3s whereas it is deubiquitinated by a number of DUBs, including USP7, USP10, USP29 and USP42. One obvious question is how these multiple DUBs are coordinated to ensure the tight, precise, and dynamic control of p53 stability and activity. Different DUBs may regulate the p53 pathway in response to different cellular stress (*e.g.,* USP29 deubiquitinates p53 in response to oxidative stress[104] whereas USP10 deubiquitinates p53 following DNA damage[102]). Different DUBs may also regulate p53 in different cellular compartments (*e.g.,* USP7 regulates p53 in the nucleus whereas Otub1 regulates p53 in the cytoplasm[110] and USP10 relocates from the cytoplasm to the nucleus to regulate p53 in response to DNA damage[102]). It is interesting to examine whether different DUBs may cooperate with each other to synergistically regulate p53 stability and activity in future studies.

Nevertheless, efforts have been made towards targeting the ubiquitin-proteasome system (UPS) for reactivating p53 in cancer therapy. For example, compounds have been developed to target the p53-MDM2 interaction such as Nutlin-3s[130], the p53-MDMX interaction such as WK298[131], or both such as RO-2443[132]. Targeting DUBs has promising potential as well. For example, the cyano-indenopyrazine derivatives small molecule compounds HBX 41108, HBX 19818, and HBX 28258[133] and P22077[134] were discovered as USP7 inhibitors. For further details about targeting the UPS for cancer therapy, please refer our recent review[135]. Future directions will aim to discover more potent and specific DUB inhibitors that can be used for cancer treatment.

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