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Review

Mdm2 and MdmX partner to regulate p53

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ABSTRACT

Mdm2 regulates the stability, translation, subcellular localization and transcriptional activity of p53 protein. Mdm2-dependent p53 inhibition is essential in regulating p53 activity during embryonic development and in adult tissues. MdmX, an Mdm2 homolog, is also essential for p53 inhibition in vivo. Recent advances in the field from biochemical and genetic studies have revealed an essential role for the MdmX RING domain in Mdm2-dependent p53 polyubiquitination and degradation. Mdm2 on its own is a monoubiquitin E3 ligase for p53, but is converted to a p53 polyubiquitin E3 ligase by MdmX through their RING–RING domain interactions. MdmX acts as an activator as well as a substrate of Mdm2/MdmX E3 complex. The insufficiency of Mdm2 for p53 polyubiquitination also demands other p53 E3 ligases or E4 factors be incorporated into the p53 degradation arena. Deubiquitinases nullify the effects of E3 actions and reverse the ubiquitination process, which permits a diverse and dynamic pattern of p53 stability control. Unsurprisingly, stress signals target MdmX to disengage the p53/Mdm2 feedback loop for timely and appropriate p53 responses to these stresses.

1. Background: the tumor suppressor p53

p53 suppresses tumor development through multiple activities including induction of growth arrest, apoptosis, senescence, and autophagy [1,2]. Beyond tumor suppression, p53 also regulates fecundity, metabolism, quiescence, and aging [3–8]. p53 remains dormant in unstressed cells owing to its inherent instability via fast degradation mechanisms. However, the short-lived p53 protein is stabilized and activated by many types of abnormal conditions including genotoxic stress, non-genotoxic such as hypoxia, low pH, heat shock and ribosomal stress, and by aberrant growth signals, among others [8–10]. Activation of p53 leads to transcriptional up-regulation of various p53 target genes [3]. To engage p53 under these various stress conditions, a complex regulatory network has evolved for precise p53 regulation. This regulatory network prevents accidental p53 activation, which is potentially lethal to many cell types, but initiates a rapid p53 response when stress signals are sensed. The key molecule in the p53 regulatory network is Mdm2, an E3 ubiquitin ligase with potentially oncogenic activity. Dynamic fine-tuning of the Mdm2-centered network dictates the proper rapidity, intensity, and duration of a p53 response, resulting in the appropriate biological outcomes [11].

p53 is modified by many types of posttranslational modifications. Among these, ubiquitination by E3 ligases has, perhaps, the most significant impact on p53 biology [12,13]. Many cellular E3 ligases capable of mediating p53 ubiquitination have been identified. The founding member of HECT domain E3 ligases that target p53 for degradation is E6AP, a protein that ubiquitinates p53 only in the presence of the human papillomaviruses E6 protein [14,15]. Mdm2 E3 ligase appears to be the physiological and primary E3 ligase regulating p53, and is thus the best studied in the field. Several recent reviews provide a comprehensive updates on our current understanding of Mdm2 [8,10,16,17]. This review will focus on recent advances in the regulation of Mdm2 E3 ligase activity by MdmX, and the significance of MdmX for p53 regulation, as summarized in Fig. 1.

2. Mdm2-dependent p53 inhibition: canceling transcription or degrading the protein

Mdm2 was initially discovered as a p53 binding protein that possesses potent inhibitory effects on p53-mediated transcription [18]. The crystal structure of an Mdm2 N-terminal fragment bound with a p53 transactivation domain peptide demonstrated that the amino acids of p53 involved in binding to the Mdm2 cleft are

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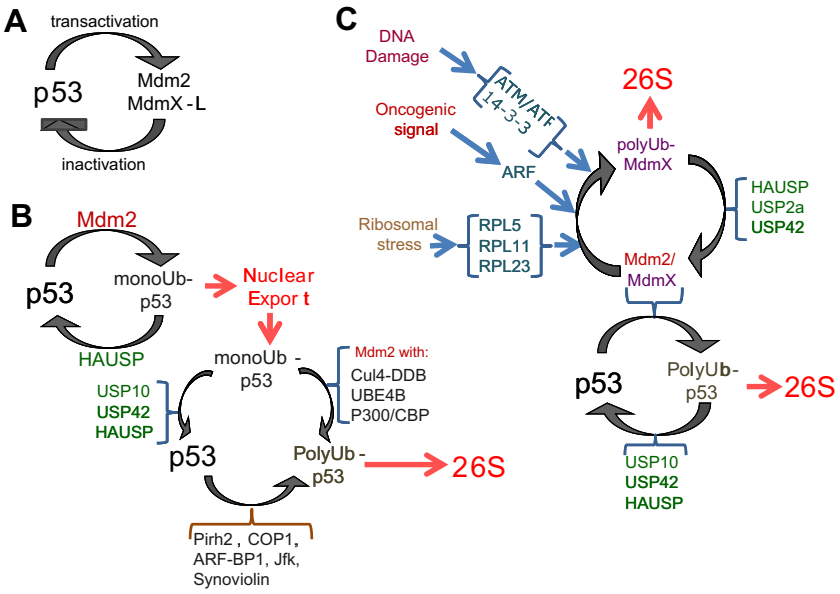

Fig. 1. Mdm2 and MdmX control p53 stability at multiple levels. (A) p53 and Mdm2 form a well-established autoregulatory feedback loop in which p53 transactivates *Mdm2* gene transcription and the subsequent increase in Mdm2 protein inactivates p53. Recently, the MdmX-L isoform has been shown to participate in a similar loop. (B) Mdm2 acting alone mediates monoubiquitination of p53 at multiple sites, facilitating p53 nuclear export to the cytoplasm. Other E3 ligases (such as Pirh2, etc.), or Mdm2 together with other E4 factors (such as UBE4B) or E4-like molecules (such as Cul4-DDB complex and p300/CBP) can polyubiquitinate p53 leading to its degradation by 26S. Deubiquitinases such as HAUSP, whose major substrates are ubiquitinated Mdm2 and MdmX, and USP10, whose major substrate is ubiquitinated p53, are involved in removal of ubiquitin moieties from their corresponding substrates and contribute to p53 stability control. (C) Mdm2/MdmX heterodimers formed via their RING domains mediate p53 polyubiquitination for efficient proteasomal degradation. Various stress signals trigger MdmX polyubiquitination by Mdm2/MdmX leading to degradation of MdmX, the activator component of Mdm2/MdmX E3 complexes, resulting in p53 stabilization and activation. Conversely, specific deubiquitinases exert dynamic regulation of p53, Mdm2, and/or MdmX by deubiquitinating these three proteins.

indeed also required for binding to transcriptional machinery [19]. Therefore, Mdm2 binding conceals the transactivation potential of p53 by forming Mdm2/p53 complexes. Later, it was found that overexpression of Mdm2 also promotes proteasomal degradation of p53 [20,21]. Using GST-Mdm2 fusion protein to reconstitute p53 ubiquitination in vitro, it was demonstrated that Mdm2 is an E3 ligase and that Mdm2 belongs to the RING family of E3 ligases [22,23]. Several other p53 E3 ligases have since been identified. However, Mdm2 appears to be essential in restricting p53 activity during embryonic development as demonstrated by mouse genetic studies [13,24,25]. In addition to p53, Mdm2 E3 ligase has other substrates such as ribosomal protein L27 that regulates p53 translation [26]. Importantly, conditional loss of Mdm2 in several adult tissues causes p53-dependent cell death accompanied by p53 protein accumulation and activation of p53 target gene expression [10,16]. Since the p53 binding domain of Mdm2 resides in the N-terminus while the E3 ligase activity resides in the C-terminus, mutation of the Mdm2 RING domain allows one to differentiate between the effects of Mdm2 on inhibiting p53 transcription and the effects on p53 degradation in vivo. Surprisingly, RING domain point mutations like Mdm2C462A cause p53-dependent lethality in a manner similar to complete deletion of the whole *Mdm2* gene [27]. This observation implies that the binding of Mdm2 to the p53 transactivation domain has little effect while the E3 ligase activity within the RING domain is sufficient to account for Mdm2 mediated p53 inhibition [28]. Subsequent to these discoveries, the Mdm2 RING domain became the centerpiece of p53 regulation in vivo.

3. MdmX as a potent activator of Mdm2-dependent p53 degradation in vitro and in vivo

The mechanism underlying Mdm2-dependent p53 degradation is complicated by the discovery of MdmX, a RING finger-containing homolog of Mdm2 [29]. Genetic studies indicate that MdmX is as essential as Mdm2 for negative regulation of p53 during embryonic development because MdmX knockout also causes p53-dependent embryonic lethality in mice [30]. Genetic analysis suggests that MdmX-mediated p53 inhibition consists of two components, one that is dependent on Mdm2 and one that is not [31]. Conditional deletion of Mdm2 causes a significant increase in p53 protein levels in MEFs, while conditional deletion of MdmX in an Mdm2 heterozygous background causes only a moderate increase in p53 protein levels. This observation has led to the conclusion that Mdm2 regulates p53 mainly through protein degradation while MdmX regulates p53 mainly via modulation of its transcriptional activity [32]. MdmX is a potent inhibitor of p53 transcriptional activity [29,33]. However, how MdmX contributes to Mdm2-dependent p53 degradation has remained controversial for many years. There are several possible reasons for this: (1) Mdm2 was originally reported to be sufficient for p53 polyubiquitination in vitro in a concentration-dependent manner [34], suggesting other factors play only minor roles in the p53 ubiquitination process; (2) MdmX has little E3 ligase activity toward p53; (3) MdmX overexpression in cell culture has generated contradictory results with regards to p53 ubiquitination and degradation (see reference in [33]).

MdmX shares low overall similarity with Mdm2 at the level of amino acid sequence. However, both proteins have a nearly identical p53 binding domain at their N-terminus and a RING domain at their C-terminus. A RING domain is a well-established E2-interacting domain that confers E3 ligase activity to RING domain-containing proteins [35]. However, RING domains can also interact with RING domains of other proteins thus forming protein heterodimers [36]. Interestingly, the Mdm2 RING domain was found capable of interacting with the MdmX RING domain [37]. Although MdmX does not possess significant E3 ligase activity towards p53, MdmX autoubiquitination does occur in vitro [38,39]. The stimulatory ef-

fects of MdmX on Mdm2-mediated p53 ubiquitination were demonstrated nicely in an in vitro reconstitution system [38,40]. Furthermore, MdmX was shown to promote Mdm2-mediated p53 ubiquitination and degradation through RING–RING interaction in cell culture systems [41–43]. However, the significance of the MdmX RING domain in Mdm2-dependent p53 degradation remains controversial.

One of our surprising observations was that GST-Mdm2 and His-tag-Mdm2 behaved differently in an in vitro reconstitution system [39]. The His-tag-Mdm2 on its own could only mediate monoubiquitination of p53 at multiple sites, which was in sharp contrast to the dose-dependent p53 polyubiquitination observed when using GST-Mdm2. After cleavage of GST from GST-Mdm2, GST-free Mdm2 was significantly compromised in its p53 polyubiquitination activity, and generated an ubiquitin-adduct pattern reminiscent of monoubiquitination of p53 at multiple sites as reported previously [44]. Inspired by the dimerization property of GST [45] and the Mdm2–MdmX heterodimers are preferred over Mdm2 homodimers [37], we reasoned that Mdm2–MdmX heterodimers might be required for robust p53 polyubiquitination. As predicted by this hypothesis, MdmX activated Mdm2 for p53 polyubiquitination in vitro in a dose dependent manner. When a mixture of recombinant MdmX and Mdm2 proteins was fractionated by gel filtration and the resulting fractions used as the E3 source for p53 ubiquitination, a strong E3 ligase activity for p53 polyubiquitination was detected only in one fraction. This fraction coincided with the peak fraction for MdmX and a sub-peak fraction for Mdm2. This rediscovery of Mdm2 as a monoubiquitin E3 led to the demonstration that MdmX as an essential activator of Mdm2 for p53 polyubiquitnation by heterodimerization through their respective RING domains [39]. These findings provided a biochemical basis for MdmX-mediated regulation of p53 in vivo [31] and for the requirement of RING domain in both MdmX and Mdm2 for efficient p53 ubiquitination and degradation [42].

Given that p53 polyubiquitination is required for proteasomal degradation and that RING domain-mediated Mdm2/MdmX interaction is required for p53 polyubiquitination, it can be predicted that MdmX RING domain mutations will cause p53-dependent embryonic lethality in mice. Consistent with this prediction, Yuan's and Lozano's groups recently demonstrated that knock-in of MdmX RING domain mutants, either a MdmxC462A point mutation or a RING domain deletion, caused a p53-dependent embryonic lethality in mice [46,47]. In both cases, the timing of embryonic lethality is around day-9.5 of gestation, which is similar to the complete MdmX knockout [30]. The day-9.5 MdmxC462A mutant embryos manifest massive p53 accumulation associated with upregulation of p53 downstream genes including *p21, bax* and *mdm2* [46]. The embryonic lethality can be rescued either by reduced p53 levels as in the p53 hypomorphic genetic background (at 15% normal level of p53) or by a genetic background containing a transcriptionally inactive p53 mutant (p53R172H) [47]. Therefore, this p53-dependent lethality is caused by two factors: (1) increased p53 protein levels; and (2) transcriptional activity of p53. Interestingly, when p53 levels are reduced to 15% of normal levels in hypomorphic p53-expressing MEFs, p53 stability is no longer under regulation by the Mdm2/MdmX complex [47]. Loss of p53 stability control by the Mdm2/MdmX complex might be the reason why hypomorphic p53 rescues the embryonic lethality of MdmX RING mutant mice. If p53 does not accumulate, then p53-dependent lethality will not occur. From biochemical point of view, this is understandable since the initial substrate concentration will significantly affect the kinetics of p53 polyubiquitination catalyzed by Mdm2/MdmX. The importance of basal p53 levels in determining p53's sensitivity to Mdm2/MdmX-mediated regulation may have additional implications: Mdm2/MdmX regulation permits a basal level of p53 to exist under non-stressful conditions.

4. Biochemical effects of MdmX on p53 ubiquitination

A precedent for a RING–RING heterodimer holoenzyme E3 ligase is the BRCA1/BARD1 complex [36]. In contrast to this complex, however, MdmX plays a dual role in the Mdm2/MdmX heterodimer of being both an activator as well as a substrate of the E3 ligase activity. This adds an additional layer of complexity to the p53/Mdm2/MdmX regulatory loop. When there is no p53, MdmX is efficiently ubiquitinated by Mdm2/MdmX. This MdmX-oriented ubiquitination protects Mdm2 from autoubiquitination-mediated degradation thus exerting a stabilizing effect on Mdm2 to maintain basal levels. However, when p53 is present, p53 becomes the preferential substrate over MdmX in the ternary complex. Moreover, when MdmX concentration exceeds a certain threshold, excess monomer MdmX might compete with p53 for polyubiquitination in vitro and stabilizes p53 in vivo [39]. This biochemical evidence may help explain earlier reports that MdmX overexpression stabilized both p53 and Mdm2 [48–50]. In most cancer cell lines, MdmX is abundantly expressed due to its constitutive expression and protein stability. Therefore, Mdm2 is the limiting factor in determining the levels of the Mdm2/MdmX holoenzyme and thus p53 degradation. Thus increasing Mdm2 levels will promote p53 degradation in a dose dependent manner. In cancer cells where basal levels of MdmX are high, however, further increases in MdmX will stabilize p53 by functioning as a competitive substrate for polyubiquitination [39,41]. In cases where MdmX basal levels are low and rate limiting relative to Mdm2, increasing MdmX expression would be expected to decrease p53 stability by augmenting its polyubiquitination. Thus, the effects of increasing MdmX expression are context dependent [39]. Another alternative mechanism underlying the complex effects of MdmX relates to the potential effect of MdmX on heterogeneity of ubiquitin chains in Mdm2-mediated ubiquitination reaction, which will be discussed later in more details.

Biochemically, how MdmX activates Mdm2 for p53 polyubiquitination is presently not known. In the ubiquitin conjugation systems, it is usually the E2 ubiqutin conjugating enzymes that determine the preferred usage of the seven lysine residues within ubiquitin thereby defining the types of polyubiquitin chains assembled on substrates. Some E2s such as UBE2K, only form K48 type polyubiquitin chains, while others such as the UbcH5 family have the potential to form K11, K27, K33, or K48 type of chains [51]. The E2 enzymes readily form homodimers in solution and homodimers are sufficient to assemble polyubiquitin chains if artificially tethered to a substrate [51]. E3 ligases constrain the E2-mediated ubiquitin conjugation reaction in two aspects: (a) the specific lysine on ubiquitin that is used and (b) the particular acceptor lysine residue modified on the substrate [52]. The acceptor lysine residues are often located in flexible loops or edges of secondary structure within substrates. Binding of E3 ligase to substrates restricts their structural flexibility and may also increase the specificity of ubiquitin chain types formed on E2 dimers that are bound to the E3s. How MdmX affects these processes as well as the processivity of Mdm2-mediated p53 ubiquitination is currently unknown. One possibility is that MdmX binding might alter the conformation of p53 or change how the E2 interacts with p53. The structure of the MdmX–p53 complex has recently been solved and it indicates that MdmX binding does alter p53 conformation. This type of conformational change is not observed in Mdm2-bound p53 [53]. Using GST-Mdm2 and GST-MdmX in an in vitro system, it was found that Mdm2 alone ubiquitinates lysine residues in the DNA binding domain of p53 while Mdm2/MdmX ubiquitinates lysine residues in the p53 C-terminus [52]. This may reflect the p53 conformational change induced by MdmX binding. Another possible mechanism may involve the effects of MdmX binding on how E2 interacts with p53, as suggested by the crystal

structure of RING heterodimers and studies with Mdm2 mutants that have weakened binding activity to UbcH5 [54,55]. The RING domains of Mdm2 and MdmX adopts a new extended E2 interface when they form heterodimers [55]. Some Mdm2 mutants are defective in p53 polyubiquitination due to their weakened E2 interaction. Interestingly, this defect can be rescued by MdmX, probably by enhancing Mdm2–E2 interaction [39,54,55]. MdmX also has an unexpected effect on influencing the ubiquitin chain types found in p53-ub adducts. The ubiquitin chain type of p53-ub adducts is K48 when ubiquitinated by GST-Mdm2 alone whereas it becomes K6, K11, and K48 types when ubiquitinated by GST-Mdm2/GST-MdmX heterodimers in the presence of UBE2D3 (UBCH5C) [52]. This heterogeneity of polyubiquitin chain types catalyzed by Mdm2/MdmX in vitro has been reported previously [38]. These findings, if confirmed with GST-free Mdm2 and MdmX in vitro and in vivo, will raise many new questions. For example, are K6, K11, K48 ubiquitin-p53 adducts degraded at equal efficiency by the 26S proteasome? What is the ratio of Mdm2 homodimers relative to Mdm2/MdmX heterodimers in cells, and how does this ratio regulate p53 stability? Does the ratio of Mdm2 homodimers to Mdm2/MdmX heterodimers change in response to stress signals? What are the E2 enzymes for Mdm2 in cells? Does the Mdm2/MdmX heterodimer use the same or different E2 enzymes?

5. Destruction of MdmX for a p53 response

Decoupling the p53/Mdm2 feedback loop is the focal point of stress signaling to p53. It appears that regulated MdmX degradation is the key to inactivation of the Mdm2/MdmX E3 ligase during a p53 response. Several laboratories have observed that DNA damage triggers accelerated MdmX degradation, which causes p53 stabilization. Interestingly, inducible degradation of MdmX is mediated by Mdm2, or more accurately by Mdm2/MdmX complex, making it an extremely efficient mechanism for decoupling the p53/Mdm2 feedback loop through self-destruction [56,57]. Furthermore, this decoupling process is regulated by the upstream DNA damage signaling kinases ATM/ATR and c-Abl, both well-established activators of the p53-dependent checkpoint response [58–62]. The phosphorylation of MdmX by these kinases stimulates recruitment of adaptor protein 14-3-3, promotes MdmX nuclear translocation [63,64], and accelerate Mdm2-dependent MdmX degradation [58,59,65]. The critical role of this regulated MdmX degradation in the p53 mediated response to DNA damage has been nicely demonstrated by the attenuated p53 stabilization in $Mdmx^{3SA}$ knockin MEFs in which the DNA damage kinase phosphorylation sites of MdmX are mutated [66]. It appears that there are two branches of signaling involved in this regulated MdmX degradation: ATM-dependent phosphorylation of MdmX [58] and c-Abl-mediated phosphorylation of Mdm2 at three putative sites that increases Mdm2/MdmX heterodimers formation after DNA damage [62]. In this case, phosphorylation of both MdmX and Mdm2 by two upstream kinases acts to switch MdmX as the preferential substrate of Mdm2/MdmX E3 ligase complex after DNA damage. Notably, it has been shown that ATM-dependent phosphorylation disrupt Mdm2 RING domain oligomerization when cellular protein samples were processed by caspase-3 that releases a fragment containing the RING domain (aa362–491) of Mdm2 and analyzed by size exclusion chromatography [67]. However, MdmX has a same capase-3 site as Mdm2. Therefore, it remains to be tested how ATM phosphorylation of Mdm2 and MdmX differentially affect formation of Mdm2 homodimers and Mdm2/MdmX heterodimers in vitro and in vivo at full length protein levels.

In addition to DNA damage, other stress responses also utilize selective degradation of MdmX as mechanism to activate p53. Oncogenic growth signals induce expression of tumor suppressor ARF, which in turn stabilizes p53 protein by binding and inhibiting Mdm2-mediated p53 polyubiquitination and degradation [68]. The N-terminal 20 amino acids of ARF bind to the acidic domain of Mdm2 and inhibit p53 ubiquitination in vitro [69]. In contrast to the effects on p53, ARF binding selectively promotes ubiquitination and degradation of MdmX [56]. In cells, ARF binding induces a second-site interaction between the central region of Mdm2 and MdmX, resulting in increased MdmX ubiquitination and subsequent degradation [70]. Hence, rather than inhibiting the intrinsic E3 ligase activity of Mdm2/MdmX, ARF acts to switch the substrate preference to MdmX thus sparing p53. Conceivably, the loss of ARF in cancer cells has dual negative effects on p53 via MdmX stabilization: (a) inhibition of p53 transactivation by MdmX and (b) enhancing p53 degradation by increasing Mdm2/MdmX E3 complex formation.

Similarly to ARF, several ribosomal proteins (RPs) including RPL11, RPL23 and RPL5 bind to the acidic domain of Mdm2. As a result, they also inhibit Mdm2-mediated p53 ubiquitination in cells [8]. Loss of homeostasis in ribosome biogenesis leads to an increase in free ribosomal proteins, which in turn serve as an emergency call to activate the p53 stress response pathway. Selective MdmX degradation is also involved in the p53 response to ribosomal stress. Perturbation of ribosome biogenesis by low doses of actinomycin D causes a significant decrease in MdmX protein. Ribosomal stress introduced by overexpressing RPL11 causes a decrease in MdmX protein accompanied by increased Mdm2-dependent MdmX ubiquitination and degradation [71]. In this case, ribosomal proteins are also substrate switchers like ARF, thus alerting the cell to ribosomal stress by activating p53.

Nucleolar protein PICT-1 is a tumor suppressor candidate whose low expression in gliomas and ovarian cancer is correlated with high malignant progression. PICT-1 was initially found to stabilize PTEN by their physical interaction. Surprisingly, knockout of *pict-1* leads to release of RPL11 from nucleolus, which in turn activates a p53 response by inhibiting Mdm2 E3 ligase activity [72]. In a recent report, 5S rRNA was found to stabilize MdmX protein. 5S rRNA binds to the RING finger of MdmX and inhibits MdmX ubiquitination by Mdm2 [73]. RNAi knockdown of 5S rRNA does not perturb ribosome assembly, nor does it affect p53 ubiquitination in vitro or p53 abundance in cells, but it does increase p53 dependent transcription [73]. Therefore, 5S rRNA might play a role to maintain constitutive levels of MdmX without serving as substrate switcher for Mdm2/MdmX.

Another significant advance in the field is the recent identification of a functional p53 responsive promoter in the MdmX gene. This makes MdmX another component of the Mdm2/p53 feedback loop. This p53 responsive promoter (P2) was found in the first intron of the human *MdmX* (*HdmX*) gene and is functional in a p53-depndent manner under various conditions [74,75]. This promoter directs production of a novel HdmX transcript that is translated into a long version of HdmX (Hdmx-L) with an extra 18 amino acids at the N-terminus. Interestingly, HdmX-L has a weakened binding affinity for p53 and thus has little effect on p53 transcriptional activity. However, HdmX-L is just as efficient as HdmX in Mdm2-mediated p53 ubiquitination and plays an important role in bringing p53 to basal levels after stress mediated activation [74].

6. Deubiquitinases as regulators of the Mdm2/MdmX E3 complex

Mdm2/MdmX E3 ligase as well as p53 can be regulated by deubiquitinases. HAUSP (USP7) was the first deubiquitinase identified for p53 and can stabilize p53 through its deubiquitination [76]. However, somatic knockout of HAUSP or embryonic knockout of HAUSP causes p53 stabilization rather than destabilization. This phenomenon is associated with loss of Mdm2 expression in HAUSP

Knockout cells, indicating that HAUSP also plays a critical role in maintaining Mdm2 stability [77,78]. It is not surprising that HAUSP also forms a complex with Mdm2 [79]. However, it is surprising that HAUSP also binds MdmX and stabilizes MdmX through deubiquitination. MdmX protein is essentially below detectable levels in HCT116/HAUSP−/− cells [80]. Therefore, p53 stabilization in HAUSP knockout cells might originate from loss of MdmX resulting in destabilization of Mdm2 protein and thus a decrease in Mdm2/MdmX E3-mediated p53 degradation. The HAUSP–MdmX interaction is disrupted by DNA damage suggesting a possible involvement of HAUSP in Mdm2/MdmX downregulation after DNA damage [80]. In contrast to HAUSP, USP2a was found to bind and specifically deubiquitinate Mdm2 and MdmX but not p53 [81,82]. Knockdown of USP2a stabilizes p53 but causes decreased expression of Mdm2 and MdmX. Treatment with DNA damaging agent cisplatin causes downregulation of USP2a in cells, which may contribute to downregulation of MdmX/Mdm2 and the eventual p53 stabilization in DNA damage response [82]. In contrast to HAUSP and USP2a, USP10 appears to specifically deubiquitinate p53 because knockdown of USP10 in HCT116/p53−/− cells does not cause reduction in Mdm2. Importantly, USP10 can be phosphorylated by ATM resulting its stabilization and nuclear translocation, a phenomenon opposite to what occurs to USP2a in DNA damaged cells [83]. Like USP10, USP42 is another p53-specific deubiquitinase and plays a role in DNA damage-induced p53 stabilization [84]. Taken together, the action of these deubiquitinases antagonizes the action of Mdm2/MdmX and other E3 ligases, which makes p53 regulation more dynamic in a context-dependent manner.

7. Other p53 E3 ligases

Although Mdm2/MdmX work together through RING–RING interactions to regulate p53 degradation, mouse studies suggest that Mdm2 and MdmX are differentially involved in p53 regulation. Knockout of Mdm2 and knock-in of an Mdm2 RING mutant kill the embryos at an earlier developmental stage (day-5.5 to 7.5) [24,27,85], while knockout of MdmX or knock-in of an MdmX RING mutant kills the embryos at day-9.5 [30,46,47]. These results suggest that at an earlier developmental stage, Mdm2 itself is sufficient to completely inhibit p53 through its RING domain without need of MdmX. Possible mechanisms underlying this stage-dependent p53 inhibition might be: (1) Mdm2-mediated monoubiquitination in the absence of MdmX drives p53 nuclear export, which is sufficient for inactivation of p53 nuclear activity; and/or (2) other existing E3 ligases or E4 factors complete the ubiquitin-dependent degradation process of the exported p53 in the cytoplasm [13]. However, at the later stage of development, the role of MdmX in Mdm2/MdmX is irreplaceable by other factors [11]. Since Mdm2 is the catalytic component of Mdm2/MdmX E3 ligase, loss of Mdm2 will be phenotypically equivalent to loss of Mdm2/MdmX as far as p53 polyubiquitination is concerned. However, the opposite is not true: loss of MdmX causes milder p53 stabilization than loss of Mdm2 [32]. This suggests that either Mdm2-mediated monoubiquitination of p53 plays a role in p53 degradation with aid of other E3 ligases, or Mdm2 homodimers that are formed in the absence of MdmX contributes to the process. In this context, discovery of cellular factors that facilitate Mdm2 homodimerization in the absence of MdmX will be the key to understanding the biochemical entities of the p53 death-insulting E3 ligases in cells. Equally important is the need for more careful analysis of other p53 E3 ligases such as Pirh2, Cul4–DDB1 E3 complexes, COP1, ARF-BP1, Synoviolin, CBP/p300(E4), UBE4B and SCF(Jfk) in well-defined systems with regard to cell type and stress specific p53 responses. Further study of these E3 ligases will help better understand the mechanisms of p53 degradation in the absence of MdmX [86–91].

With regard to p53 polyubiquitination, there is confusion in interpreting p53 polyubiquitination versus multiple monoubiquitination in the literature. Using wild type and methylated ubiquitins in an in vitro p53 ubiquitination reaction by Mdm2, Lai et al. demonstrated that seven distinct new p53 bands were recognizable on an SDS–PAGE gel, indicating seven primary ubiquitination sites on p53 [44]. A polyubiquitin chain of 4-ubiquitin length can serve a degradation signal for 26S proteasomes [92]. Based on the calculation, the degradable polyubiquitinated p53 species should appear in a smearing pattern above 138 kDa on SDS–PAGE gels. Therefore, we suggest that polyubiquitinated p53 region should be labeled at least above 138 kDa on a SDS–PAGE gel in future studies. In addition to p53 polyubiquitination by the Mdm2/MdmX complex, Cul4–DDB1 complexes, UBE4B and p300/CBP are worthy of discussion. Cullin4 (Cul4)-containing E3 ligases are multi-subunit E3 complexes consisting of Cullin4A or 4B (scaffold), DDB1 (linker subunit), WD40 proteins (substrate receptor) and ROC1 (RING-domain subunit). Cul4–DDB1complexes are involved in nucleotide excision repair and are responsible for DNA damage-induced degradation of CDT1, a DNA replication licensing factor, in the presence of L2DTL/CDT2 and PCNA [93]. Cul4 scaffolds interact with more than 50 WD40 proteins and thus may target numerous substrates for ubiquitin-dependent regulation [94]. Interestingly, Cul4–DDB1-containing E3 ligases are capable of mediating p53 polyubiquitination in an Mdm2-dependent manner [95]. Both p53 and Mdm2 can be detected in the Cul4–DDB1 E3 complexes and knockdown of each component of these E3 complexes cause p53 accumulation in several cell lines. DNA damage or UV irradiation leads to dissociation of p53 from Cul4–DDB1 E3 complexes implying that this E3 activity may be involved in maintaining fast turnover of p53 in unstressed cells. Surprisingly, the Cul4A-specific immunoprecipitates from Mdm2/p53 double-null MEFs lacks this activity in vitro, suggesting that Cul4–DDB1 complexes do not possess intrinsic E3 ligase activity for p53 polyubiquitination. However, the Cul4A-specific immunoprecipitates from wild type MEFs or Mdm2-overexpressing Mdm2/p53 double-null MEFs can promote extensive p53 polyubiquitination in vitro, indicating Mdm2-dependence of this process. Therefore, Cul4–DDB1 complexes act as E4 factors in this case. It was proposed that PCNA is the bridging molecule that brings Mdm2 into the Cul4–DDB1 complexes. However, how signaling events regulate Mdm2–PCNA interaction and whether the RING domain of Mdm2 is required in this process merit further investigation. Although deletion of DDB1, the linker component of Cul4-based E3 ligases, in brain causes p53-dependent apoptosis, thus supporting potential roles of this E3 family in p53 regulation in vivo, it is yet to be determined whether this is a direct effect of the Cul4–DDB1 E3 complexes or an indirect effect of DNA damage-signaling observed in DDB-null tissues [96].

Similar to Cul4–DDB1 complexes, the mammalian homolog of yeast ubiquitin chain assembly factor UBE4B has been shown to promote p53 polyubiquitination and cellular degradation in the presence of Mdm2 [97]. In fact, UBE4B is a member of U-box E3 ligase family [98], possessing intrinsic U-box-dependent E3 ligase activity toward p53. However, p53 polyubiquitination by UbE4B itself is low but is greatly enhanced in the presence of Mdm2. Knockdown of UBE4B reduced polyubiquitinated p53 species in cells was accompanied by accumulation of multi-monoubiquitinated p53, indicating a major role of UBE4B as an E4 factor in the p53 ubiquitination process. Of note, the N-terminal part of p300 and CBP acetyltransferases has been shown to possess similar activity to UBE4B [90,99]. p300 or CBP specific immunoprecipitates strongly promotes p53 polyubiquitination of Mdm2–p53 complex in vitro. Moreover, knockdown of either p300 or CBP results in p53 accu-

mulation in cells. Interestingly, this E4 activity only exists in the cytosolic CBP complex but not in the nuclear CBP complex. This novel activity assigns a new role to p300/CBP in p53 regulation, opposing its nuclear acetyltransferase effect on p53 through acetylation of p53 c-terminal lysine residues [100,101]. Many interesting questions arise from this observation. For example, is this E4 activity intrinsic to p300/CBP proteins? Are the putative Zn-finger-like domains a structural requirement of p300/CBP for this E4 activity? Is this activity regulated by DNA damage signaling or by associated factors in cytoplasm? Is RING domain of Mdm2 required in this process? Future investigation using recombinant CBP/p300 proteins rather than CBP/p300-immunoprecipitates in an in vitro system will provide answers to these questions.

As summarized in Fig. 1, recent evidence from in vitro biochemical analysis and in vivo mouse models has established an essential role of the MdmX RING domain in p53 inhibition through controlling p53 stability. Several p53 signal transduction pathways utilize MdmX downregulation to launch a p53 response. Other E3 ligases and E4 factors contribute to p53 stability control when Mdm2 is not sufficient for p53 polyubiquitination. Actions of different deubiquitinases enable a dynamic regulation of the p53 levels. Further investigation of RING–RING interaction of Mdm2 and MdmX should lead to better understanding of Mdm2/MdmX E3 ligase regulation. Undoubtedly, studies on functional compensation of Mdm2 by other E3 ligases will reveal more specific details of the p53 degradation process in cells.

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