

# p53 regulation

## Teamwork between RING domains of Mdm2 and MdmX

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**Key words:** p53, Mdm2, MdmX, Mdm2/MdmX, MDM4, RING domains, protein stability, ubiquitination, lethality, knockout, knockin

p53 is a major tumor suppressor frequently inactivated through direct gene mutation and alternative mechanisms, including overexpression of Mdm2 and MdmX. Both Mdm2 and MdmX are essential for negative regulation of p53 in vivo in a mutually dependent manner. The RING domain-dependent E3 ligase activity of Mdm2 has been shown to be essential for negative regulation of p53. The prevailing model has dubbed MdmX as an inhibitor of p53 transcriptional activity through direct binding of its N-terminal domain to p53. However, recent findings established an essential role of the RING domain of MdmX in p53 degradation in vitro and in vivo. Biochemically, Mdm2 on its own is a monoubiquitination E3 ligase; however, MdmX can convert Mdm2 into a polyubiquitination E3 ligase necessary for p53 proteasomal degradation in cells, through their RING-RING interaction. While Mdm2 is the catalytic component of Mdm2/MdmX E3 complex, MdmX is both the activating component and a substrate of the holoenzyme. Knock-in of RING mutant *MdmX* in mice causes p53-dependent embryonic lethality in a similar manner to knockout of *MdmX* whole gene. These recent advancements in the field assigned an essential role of the RING domain of MdmX in negative regulation of p53 in vivo, just like Mdm2 RING domain, through p53 degradation.

### Introduction

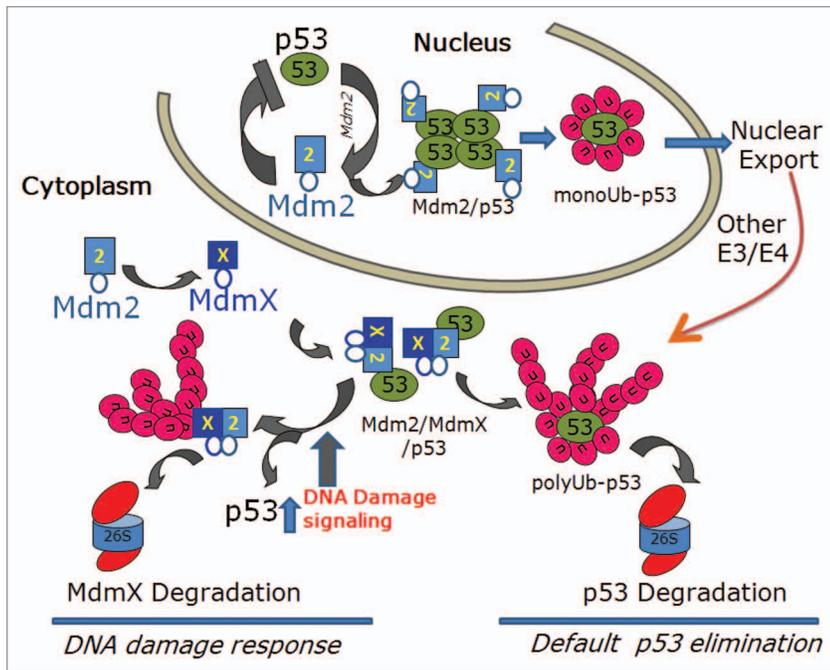
Tumor suppressor *p53* gene is mutated in more than 50% human cancers. Alternative mechanisms, including overexpression of Mdm2 and MdmX, are responsible for functional inactivation of tumor suppressor p53 in the rest of human cancer without *p53* gene mutation.<sup>1</sup> As a checkpoint protein, p53 is activated by various stressful signals via a disruption of a p53/Mdm2 feedback loop, including destruction of MdmX protein. p53 activation is accompanied by protein stabilization and increased transcriptional activity via many types of posttranslational modifications, including phosphorylation, acetylation, sumoylation, ubiquitination, deubiquitination and methylation.<sup>2</sup> These modifications individually serve to fine-tune the p53 response to certain stressful conditions, as revealed by p53 modification site mutant knock-in

mice. Genetic studies have shown that Mdm2 and MdmX are two key regulators of p53 activity in vivo.<sup>3</sup> p53 activity is precisely controlled by multiple mechanisms and feedback loops,<sup>4-20</sup> because activation of p53 can lead to apoptosis, quiescence and senescence.<sup>21-31</sup> Furthermore, the ability of p53 to choose between quiescence and senescence is determined by its ability to inhibit mTOR.<sup>32-40</sup> Therefore, p53 can affect aging in opposite ways by inducing arrest and by affecting mTOR and metabolism.<sup>41-51</sup>

### The RING Domain of Mdm2 as the Key in p53 Inhibition In Vivo

Mdm2 negatively regulates p53 through a default mechanism. Mdm2 binds to and inhibits p53's transcriptional activity and also promotes its ubiquitin-dependent proteasomal degradation by serving as a RING domain E3 ligase for p53.<sup>52-54</sup> Among many other known E3 ligases of p53, Mdm2 appears to be the master E3 ligase, as demonstrated by mouse genetics.<sup>3,55</sup> However, the mechanism by which Mdm2 negatively regulates p53 was complicated by the discovery of MdmX, a RING finger containing homolog of Mdm2.<sup>56</sup> Genetic studies indicate that MdmX is equally important as Mdm2 in the negative regulation of p53 during embryonic development, because *MdmX* knockout in mice causes p53-dependent embryonic lethality, as *Mdm2* knockout does.<sup>57</sup> Genetic analysis also suggested that MdmX-mediated p53 inhibition consists of two components: Mdm2-dependent and Mdm2-independent.<sup>58</sup> However, using conditional knockout strategies, it was found that homozygous deletion of *Mdm2* causes a significant increase in p53 protein levels in MEFs, contrasting with a moderate increase in p53 protein levels in MEFs with homozygous deletion of *MdmX* in an *Mdm2* heterozygous background. This led to the conclusion that Mdm2 regulates p53 primarily by regulating p53 protein stability, while MdmX regulates p53 primarily through alternative mechanisms.<sup>59</sup> Although it has been shown with knock-in mice of Mdm2-binding point mutant p53 (p53QS) that Mdm2-p53 binding is required for p53 inhibition by Mdm2,<sup>60</sup> the p53-dependent lethality of the knock-in mice of the enzyme-dead Mdm2 RING domain mutant indicated that Mdm2 RING finger is essential for p53 inhibition, and binding of Mdm2 alone to p53 is not sufficient for p53 inhibition.<sup>61</sup> Therefore, Mdm2 RING domain mediated p53 degradation became the centerpiece of the p53 inhibition during development and probably also in adult tissues.

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Submitted: 11/03/11; Accepted: 11/04/11  
<http://dx.doi.org/10.4161/cc.10.24.18662>



**Figure 1.** p53 regulation by Mdm2 and MdmX. Under normal conditions, Mdm2, as a product of p53 target gene, inhibits p53 activity, thus forming a p53/Mdm2 autoregulatory feedback loop. Mdm2 can bind to p53 and alone mediates multiple monoubiquitination, which promotes p53 nuclear export. Other E3 ligases and E4 factors can further act on monoubiquitinated p53 for polyubiquitination and degradation in cytoplasm. Mdm2/MdmX heterodimers formed via RING domains of the two proteins mediate p53 polyubiquitination for efficient proteasomal degradation. DNA damage signaling triggers MdmX polyubiquitination by Mdm2/MdmX, leading to physical removal of MdmX, the activator component of Mdm2/MdmX E3 complex, resulting in p53 stabilization and activation.

### The Key Role of MdmX RING Domain in p53 Polyubiquitination In Vitro

It has been well-established that MdmX can inhibit p53 transcriptional activity by direct p53 binding.<sup>56</sup> However, how MdmX was integrated into the centerpiece of Mdm2-E3-dependent degradation of p53 remained unsolved until recently. This was because of opposing results reported from cell culture systems with MdmX overexpression (reviewed in ref. 62). In contrast to several observations that the MdmX overexpression stabilizes p53, Yuan's group and others have demonstrated that RING-RING mediated interaction can promote Mdm2-mediated p53 ubiquitination and degradation using in vitro and cell culture systems.<sup>63-66</sup> Another factor contributing to the confusion is that Mdm2 had been accepted to be self-sufficient for p53 polyubiquitination in a concentration-dependent manner in in vitro assays.<sup>67</sup> Therefore, the significance of the positive role of MdmX RING domain in Mdm2-dependent p53 degradation had not been pursued enthusiastically, thus a consensus has not been reached in the field for many years.

An unexpected result from an in vitro reconstitution system led to the discovery of the biochemical role of MdmX in Mdm2-mediated p53 ubiquitination.<sup>68</sup> It revealed that Mdm2 on its own can only mediate multiple monoubiquitination of p53 even at a high concentration of 900 nM, which was in sharp contrast to the dose-dependent p53 polyubiquitination by GST-Mdm2. It

further revealed that the GST-tag in GST-fusion Mdm2 causes artificial activation of Mdm2 E3 for p53 polyubiquitination, especially at higher concentrations. After cleavage of GST from GST-Mdm2, GST-free Mdm2 is significantly compromised only in activity for polyubiquitination of p53. Using wild type ubiquitin and lysineless ubiquitin, it was concluded that non-GST-Mdm2 only mediates multiple monoubiquitination of p53. These findings are reminiscent of a previous report using very low concentrations of GST-Mdm2 (3 nM).<sup>69</sup> In view of the dimerization property of GST,<sup>70</sup> it was reasoned that Mdm2 dimerization might be a critical step for its activation as a holoenzyme E3 ligase for p53 polyubiquitination. Then MdmX became the first candidate for the cellular activator of Mdm2, since it is the physiological dimerization partner of Mdm2 in cells.<sup>71</sup> As expected, MdmX could activate Mdm2 E3 ligase activity for p53 polyubiquitination in a dose-dependent manner. The gel filtration experiment using a mixture of recombinant MdmX and Mdm2 proteins further confirmed that the strong E3 ligase activity for p53 polyubiquitination only resides in a fraction of the MdmX peak with Mdm2 sub-peak, which is in sharp contrast to the weak activity of the Mdm2 peak fraction with an MdmX minor peak. These data establish that MdmX is an essential activator of Mdm2, able to convert Mdm2 into a p53 polyubiquitination E3 ligase

in vitro, a process requiring RING-RING interaction between Mdm2 and MdmX.<sup>68</sup> This biochemical evidence is mirrored in an early report from Yuan's group that RING domain-mediated interaction between MdmX and Mdm2 is a requirement of the E3 ligase activity of Mdm2 using Mdm2/MdmX/p53 triple-knockout MEFs.<sup>64</sup> These results brought up an issue that caution should be paid when GST-fusion proteins are used in biochemical reactions involving homo- or hetero-dimerization, because GST-mediated dimerization can have a significant impact on the reactions.

### The Dual Role of MdmX in Mdm2/MdmX E3 Complex Explains Opposing Observations

The Mdm2/MdmX heterodimer forms a holoenzyme E3 ligase for p53 polyubiquitination and subsequent proteasomal degradation. This is reminiscent of activation of BRCA1 E3 ligase activity by BARD1 heterodimerization.<sup>72</sup> Importantly, MdmX plays a dual role in the Mdm2/MdmX complex, on one hand, being an activator of the E3 ligase and, on the other, the substrate of Mdm2/MdmX E3 ligase. This adds a layer of complexity to the effects of MdmX on the p53/Mdm2 loop, because MdmX can compete with p53 for accepting ubiquitin moiety from E2 at higher concentrations.<sup>68</sup> Owing to this property, it is now easier to understand that in some early reports, MdmX

overexpression actually stabilizes p53 and Mdm2 proteins.<sup>73-75</sup> This is because, unlike low levels of Mdm2 protein in most of the cell lines, the abundance of MdmX is usually high due to its constitutive expression and protein stability. So, Mdm2 is the only limiting factor in forming Mdm2/MdmX holoenzymes under normal conditions. Therefore, overexpression of Mdm2 will dose-dependently promote p53 degradation in most cases. However, simply overexpressing MdmX on top of high levels of endogenous MdmX will dramatically alter the stoichiometry between Mdm2 and MdmX, which allows formation of excessive inactive monomers or homodimers of MdmX competing for substrate p53 binding. This notion is supported by the observation that p53 is the preferential substrate for polyubiquitination by Mdm2/MdmX within a range of MdmX concentrations, but above that range, p53 polyubiquitination is declined in vitro, and p53 degradation is weakened in vivo.<sup>68</sup> Thus, the outputs of MdmX in cells are difficult to measure in overexpression experiments, unless p53, Mdm2 and MdmX expression levels are carefully titrated to test one factor at a time with the concentrations of the other two factors fixed.<sup>68</sup> Given its activator role in Mdm2/MdmX complex, the regulated degradation of MdmX triggered by the DNA damage signal now makes great sense. This stress-induced removal of an E3 activator allows a rapid inactivation of the E3 ligase activity of the Mdm2/MdmX complex, which heralds p53 accumulation in the cytoplasm and then nuclear translocation in response to DNA damage. Moreover, degradation of MdmX by Mdm2 or, more accurately, by Mdm2/MdmX E3, provides an extremely efficient mechanism for inactivation of Mdm2/MdmX activity.<sup>76</sup> Regulation of this process by DNA damage signaling kinases ATM/ATR as well as recruitment of 14-3-3 indicates the key role of MdmX in preventing p53 from activation under normal conditions.<sup>77-80</sup>

### The Essential Role of RING Domain of MdmX in p53 Degradation In Vivo

The prediction from the biochemical or molecular biology findings is that a RING domain MdmX mutant will cause p53-dependent embryonic lethality in mice, given that p53 polyubiquitination/proteasomal degradation depends on RING domain-mediated Mdm2/MdmX interaction. Consistent with this prediction, two recent reports from Yuan's and Lozano's groups demonstrated that knock-in of MdmX RING domain mutants either as point mutant MdmxC462A or as RING domain deletion in mice caused a p53-dependent embryonic lethal phenotype.<sup>81,82</sup> In both cases, the time point of embryonic lethality was embryonic day 9.5, which is similar to knockout of the whole gene of *MdmX*.<sup>57</sup> These results suggest that the RING domain of MdmX carries the whole function of MdmX protein in p53 inhibition during development. The day 9.5 embryos of MdmxC462A mutants manifested massive p53 accumulation associated with upregulation of p53 downstream genes, including *p21*, *bax* and *mdm2*.<sup>81</sup> Therefore, this p53-dependent lethality is caused by two factors: (1) the increased protein levels of p53 and (2) the transcriptional activity of p53, because the lethality can be rescued either by hypomorphic p53 background (at 15%

normal level of p53) or by a transcriptionally inactive mutant *p53* (p53R172H) background in the presence of elevated mutant p53 levels.<sup>82</sup> Surprisingly, when p53 levels are reduced to 15% of normal levels, p53 stability is no longer under the regulation of the Mdm2/MdmX complex.<sup>82</sup> This disconnection between p53 stability control from the Mdm2/MdmX complex might be the reason why hypomorphic p53 can rescue the p53-dependent lethality of the MdmX RING mutant: because if p53 is not accumulated, p53-dependent lethality will not be incurred. This phenomenon is easily understood from a biochemical point of view: a sufficiently high initial concentration of substrate (in this case, apparently at least > 15% of normal p53 levels) is required for an efficient enzymatic reaction to take place in cells (in this case, p53 polyubiquitination catalyzed by Mdm2/MdmX). The importance of basal levels of p53 in p53's sensitivity to Mdm2/MdmX may have further implications in that MdmX, by serving as a substrate of Mdm2/MdmX and competing with p53 for polyubiquitination, might act in an opposite way to stabilize p53 and maintain a stable basal level of p53 under non-stressful conditions, a notion yet to be tested. In this sense, MdmX serves as the critical equilibrium changer.

### The p53 E3 Ligase: Mdm2/MdmX and Beyond

If the mutual dependence of Mdm2 and MdmX as a p53 polyubiquitination E3 ligase is true, and only Mdm2/MdmX complex is critical to promote p53 degradation during development, then, the prediction will be that the p53-dependent lethality after knockout of *Mdm2* or *MdmX* or knock-in of *Mdm2 RING mutant* or *MdmX RING mutant* will follow the same time point of embryonic lethality. But this was not the case. Knockout of *Mdm2* and knock-in of *Mdm2 RING mutant* kill the embryos at the earlier developmental stage of Day 5.5–7.5,<sup>61,83,84</sup> while knockout of *MdmX* or knock-in of *MdmX RING mutant* kill the embryos at the later stage of day 9.5.<sup>57,81,82</sup> These observations imply that Mdm2-mediated p53 inhibition at an earlier developmental stage depends on its RING domain but does not need MdmX. The possible mechanisms underlying this stage of p53 inhibition might be (1) Mdm2-mediated monoubiquitination drives p53 nuclear export which is sufficient for inactivation of p53 nuclear activity or (2) on top of nuclear exporting of p53 by Mdm2-catalyzed monoubiquitination, other E3 ligases or E4 factors can complete the ubiquitin-dependent degradation process in the cytoplasm. However, at a later stage of development, the role of MdmX in Mdm2/MdmX is irreplaceable by other factors. Another similar argument concerns why homozygous deletion of Mdm2 causes a more significant increase in p53 protein levels in MEFs than homozygous deletion of *MdmX* in an *Mdm2* heterozygous background, given that both molecules work as a team for p53 degradation.<sup>59</sup> Interpretation of this observation has to take into consideration again “that Mdm2 is the catalytic half of the Mdm2/MdmX complex, which can mediate monoubiquitination of p53 without MdmX;”<sup>68</sup> several other p53 E3 ligases and E4 factors exist in cells,<sup>3,85</sup> and MdmX itself is unable to initiate p53 degradation in a significant way and can only boost Mdm2 as a polyubiquitination E3 ligase. In my opinion, the collective action

of Mdm2 and other E3 ligases or E4 factors contributes more significantly to p53 degradation when E3-incompetent MdmX protein is absent in *Mdmx*-knockout MEFs, and this nature of MdmX will be translated into a mild effect on p53 accumulation in *Mdmx*-knockout cells in comparison with *Mdm2*-knockout cells. Taking other factors into consideration, p53 regulation by Mdm2 and MdmX with regards to p53 ubiquitination and degradation is summarized in **Figure 1**. Of note, the cooperative effects of Mdm2 with other p53 E3 ligases have not been well-explored so far. The influence of other E3 ligases and E4 factors may be quite significant in p53 stability control in adult tissues in a context-dependent manner.

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Taken together, the recent evidence from in vitro biochemical analysis and in vivo mouse models established an essential role of the RING domain of MdmX in p53 inhibition in vivo through p53 stability control. This is a significant advancement, and the RING-RING mediated interaction of Mdm2 and MdmX merits further investigation in the field.

## Acknowledgments

This work was supported in part by Elsa U. Pardee Foundation (X.W.). The author thanks John J. McGuire and Benjamin E. Paluch for critical reading of the manuscript.

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