

Perspective

ATM-Mediated Phosphorylations Inhibit Mdmx/Mdm2 Stabilization by HAUSP in Favor of p53 Activation

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Received 06/29/05; Accepted 06/29/05

Previously published online as a *Cell Cycle* E-publication:
<http://www.landesbioscience.com/journals/cc/abstract.php?id=1981>

KEY WORDS

Mdm4, Mdmx, Mdm2, USP7, HAUSP, 14-3-3, p53, ATM, deubiquitination, DNA damage

ACKNOWLEDGEMENTS

We wish to thank Koji Okamoto for sharing unpublished results.

ABSTRACT

The p53 tumor suppressor protein has a major role in protecting genome integrity. Under normal circumstances Mdmx and Mdm2 control the activity of p53. Both proteins inhibit the transcriptional regulation by p53, while Mdm2 also functions as an E3 ubiquitin ligase to target both p53 and Mdmx for proteasomal degradation. HAUSP counteracts the destabilizing effect of Mdm2 by direct deubiquitination of p53. Subsequently, HAUSP was shown to deubiquitinate Mdm2 and Mdmx, thereby stabilizing these proteins. The ATM protein kinase is a key regulator of the p53 pathway in response to double strand breaks (DSBs) in the DNA. ATM fine-tunes p53's response to DNA damage by directly phosphorylating it, by regulating additional post-translational modifications of this protein, and by affecting two p53 regulators: Mdm2 and Mdmx. ATM directly and indirectly induces Mdm2 and Mdmx phosphorylation, resulting in decreased activity and stability of these proteins.

We recently provided a mechanism for the reduced stability of Mdm2 and Mdmx by showing that ATM-dependent phosphorylation lowers their affinity for the deubiquitinating enzyme HAUSP. Altogether, the emerging picture portrays an elaborate, but fine-tuned, ATM-mediated control of p53 activation and stabilization following DNA damage. Further insight into the mechanism by which ATM switches the interactions between HAUSP, Mdmx, Mdm2 and p53, to favor p53 activation may offer new tools for therapeutic intervention in the p53 pathway for cancer treatment.

MDMX AND MDM2, TWO KEY REGULATORS OF THE p53 PATHWAY

The p53 tumor suppressor gene encodes a sequence-specific transcription factor whose activity is either disabled or attenuated in the vast majority of human cancers.¹⁻³ p53 transcriptionally activates a vast number of target genes, resulting in various biological outcomes such as cell cycle arrest and apoptosis.^{1,3,4} One of the best-characterized targets of p53 is the *MDM2* gene; p53 binds two adjacent p53-responsive elements within the second promoter, thereby promoting transcription of the *MDM2* gene.⁵⁻⁷ Under normal circumstances, p53 is tightly and negatively regulated through interaction with the protein product of this gene, Mdm2. The Mdm2 protein is an E3 ubiquitin ligase that mediates ubiquitin-dependent proteasomal degradation of p53.^{8,9} The binding between Mdm2 and the N-terminal domain of p53 also blocks the interaction of transcriptional coactivators with p53.¹⁰ This autoregulatory negative feedback loop, in which Mdm2 expression is induced by p53, ensures proper p53 activity in normal cells. It also implies that uncontrolled, increased expression of Mdm2 may result in inappropriately reduced p53 function. It has been shown that 5–10% of all human tumors overexpress Mdm2 due to either gene amplification or transcriptional- or post-transcriptional mechanisms.^{5,11} In most of these cases the p53 gene is wild type, presumably because Mdm2 overexpression alleviates the selective pressure for direct mutational inactivation of the p53 gene.

Another important regulator of p53 is the Mdmx protein, originally identified in a screen for p53 binding proteins¹² and later found also in a yeast two-hybrid screen as an Mdm2 binding protein.^{13,14} Coimmunoprecipitation experiments showed the existence of a trimeric complex containing Mdmx, p53 and Mdm2.¹⁵ Comparison of the Mdmx and Mdm2 amino acid sequences revealed their structural homology (further reviewed by Marine and Jochemsen (2004)).¹⁶ Mdmx overexpression inhibits p53-stimulated transcription in transfection experiments, dependent on its p53 binding domain and, accordingly, downregulation of Mdmx upregulates p53-dependent transcription.^{12,17} Furthermore, Mdmx might affect p53 function indirectly by promoting Mdm2's activity as an E3 ligase of p53.^{18,19} Accordingly, Mdmx-null mouse embryonic fibroblasts exhibit elevated expression

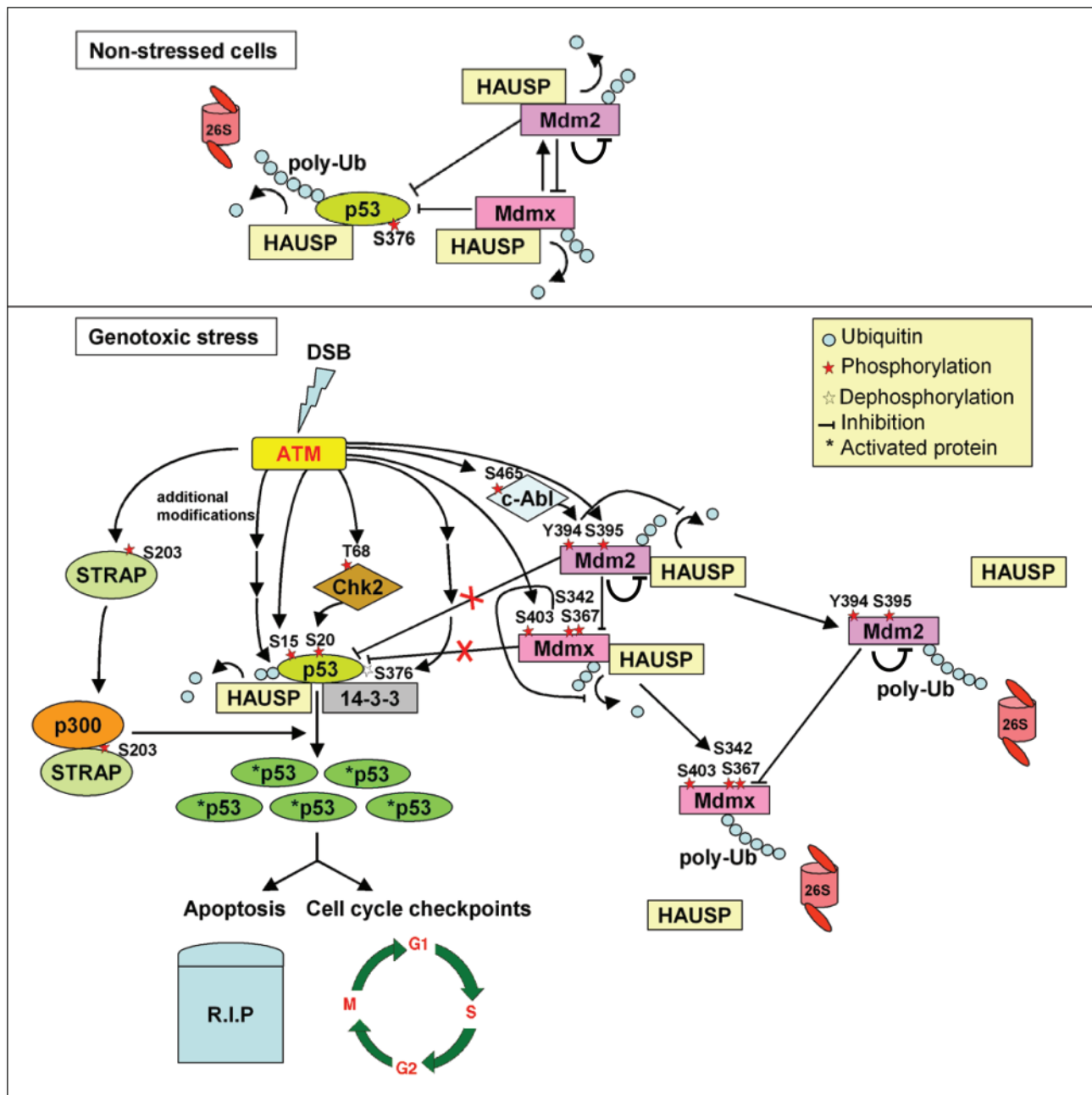


Figure 1. Activation of the p53 pathway in response to DSBs. In non-stressed cells p53, Mdmx and Mdm2 levels are regulated by a balance between ubiquitination by Mdm2 and deubiquitination by HAUSP. Mdmx stabilizes Mdm2 by inhibition of autoubiquitination, whereas Mdm2 mediates the degradation of both p53 and Mdmx. Mdm2 as well as Mdmx inhibit the transcriptional transactivation by p53. Upon DNA damage ATM is activated and mediates direct and indirect post-translational modifications of p53. Binding of 14-3-3 proteins is facilitated by ATM-mediated de-phosphorylation of p53. A safeguard mechanism exists to ensure proper p53 activation by inactivating p53's inhibitors, Mdm2 and Mdmx. Phosphorylations of Mdm2 and Mdmx attenuate their interaction with the ubiquitin protease HAUSP, destabilizing both of them. Furthermore, ATM-mediated phosphorylation of Strap increases its interaction with p300, which leads to increased p300-stimulated acetylation of p53. In this manner, ATM activates p53 while inhibiting its negative regulators. Activation of p53 protein mediates cell cycle checkpoint pathways or alternatively to an apoptotic pathway.

of p53 target genes.^{20,21} The physiological importance of Mdmx in p53-regulation has been highlighted by the observation that the absence of Mdmx causes embryonic lethality in mice, which is rescued by p53 deletion.²⁰⁻²² Since *Mdm2*-knockout mice are also embryonic lethal in a p53-dependent manner, Mdmx and Mdm2 cannot substitute for one another in the regulation of p53.^{23,24} Studying a large series of gliomas initially disclosed evidence of an important role for Mdmx in p53 regulation in oncogenesis. The *MDMX* gene

was found to be the common amplified gene in a subset of gliomas, correlating with wild type p53 expression.^{25,26} Similarly, overexpression or aberrant expression of Mdmx, found in about 30% of human tumor cell lines, is correlated in most cases with the presence of wild-type p53.²⁷ Lastly, we recently showed that the *MDMX* gene is overexpressed in a significant percentage of various human tumors, all of which retained wild-type p53.¹⁷ Similarly, Mdmx overexpression allows primary mouse embryonic fibroblast immortalization in the

absence of mutations/loss of p53 or p19ARF, and leads to neoplastic transformation in combination with H-ras^{V12}.¹⁷ Collectively, the data suggest that overexpression of Mdmx overcomes the necessity for p53 mutation or deletions for progression of human tumors.

p53 ACTIVATION UPON DOUBLE STRAND DNA BREAKS

Upon DNA damage a complex network of responses is initiated.^{28,29} One branch recognizes and repairs the damaged DNA, while numerous other pathways activate responses related to many aspects of cellular metabolism. A prominent response is activation of the damage-induced cell cycle checkpoints.³⁰ Activation and stabilization of the p53 protein play an important role in one of the major pathways that control the G₁/S checkpoint. The DNA damage response is evoked to its fullest extent by double strand breaks (DSBs).^{31,32} The primary regulator of the DSB response is the nuclear protein kinase ATM. DSBs induce immediate auto-phosphorylation of ATM, which then rapidly phosphorylates numerous substrates, thereby modulating their activity or stability and affecting the pathways in which they function.^{31,33,34} Here we will focus on the ATM-mediated activation of the p53 pathway (For a schematic representation of the ATM-dependent regulation of p53 after DSBs as discussed here, see Fig. 1). In normal growing, untransformed cells p53 has a short half-life—about 30 minutes. Following DSBs, p53 becomes rapidly stabilized and functionally activated as a consequence of several post-translational modifications. The p53 protein plays a major role in cellular stress responses by initiating an important protective mechanism to allow repair of DNA damage or to remove cells from the population by means of apoptosis. Activated ATM rapidly phosphorylates p53 on Ser15, and this phosphorylation was reported to enhance its transcriptional activity.^{35,36} The ATM-related kinase, ATR, maintains phosphorylation of Ser15 at later stages during the DNA damage response. Upon Ser15 phosphorylation, p53 is phosphorylated on Thr18, which might affect the Mdm2/p53 interaction.³⁷⁻³⁹ Furthermore, ATM (and later, probably ATR) orchestrates the phosphorylation of p53 on Ser20, which was reported to increase affinity for the transcriptional coactivator p300.^{40,41} Not only that, ATM further controls the phosphorylation of p53 on several other residues indirectly by activating several kinases, such as Chk1, Chk2 and HIPK2.⁴²⁻⁴⁷ Interestingly, upon DNA damage the p53 protein is also subjected to de-phosphorylation on Ser376 in an ATM-dependent fashion.⁴⁸ The de-phosphorylation of Ser376 creates a consensus 14-3-3 binding site and leads to the association of p53 with 14-3-3. This, in turn, elevates p53's sequence-specific DNA binding. The phosphorylation of p53 cofactors is yet another way by which the ATM kinase stimulates the activation of p53. It has been reported that the cofactor Strap is phosphorylated by ATM on Ser203, resulting in increased nuclear localization and association with p300.⁴⁹ Phosphorylation of Strap results in increased acetylation and activation of p53, suggesting that Strap/p300 complexes are recruited to p53 to induce its acetylation. In conclusion, ATM regulates many post-translational modifications of p53, ensuring a proper p53 response to DSBs. Besides phosphorylation and acetylation, the p53 protein is also methylated, NEDDylated, SUMOylated, and modified by Pin1 upon DNA damage.⁵⁰⁻⁵⁵ It would be interesting to find out whether ATM-mediated signaling also influences the efficiency of these p53 modifications upon DNA damage.

ATM INHIBITS THE INHIBITORS OF p53

ATM not only coordinates modifications of p53 but also influences the cellular abundance of Mdm2 and Mdmx, which is now emerging as an important step in p53 activation.^{56,57} ATM directly phosphorylates Ser395 on Mdm2, which impairs nuclear export and degradation of p53.^{58,59} In addition, ATM mediates indirect phosphorylation of Mdm2 on Tyr394 via the c-Abl kinase.⁶⁰⁻⁶² It has been suggested that the phosphorylation of Mdm2 by c-Abl reduces the Mdm2-mediated ubiquitination and nuclear export of p53.⁶² Lastly, Okamoto and coworkers have shown that ATR directly phosphorylates Mdm2 on Ser407 in response to replication block, and this phosphorylation too was proposed to reduce the ability of Mdm2 to degrade p53 after DNA damage.⁶³ These results could explain, at least partly, the impaired degradation and inhibition of p53 activity by Mdm2 upon DNA damage. Further explanation comes from the experiments performed by Stommel and Wahl (2004), showing that phosphorylation of Mdm2 by DNA damage-induced PI3KKs, including the S395 phosphorylation, temporarily destabilizes the Mdm2 protein.⁵⁶ Incubation with wortmannin, a general inhibitor of PIKK kinases, prevented the DSB-induced destabilization of Mdm2. Importantly, their experiments also indicated that destabilization of Mdm2 is essential for proper activation of p53.

We recently showed that ATM-mediated signaling also regulates the stability of Mdmx.⁵⁷ Knowing that Mdmx is degraded in an Mdm2-dependent manner following DNA damage,⁶⁴⁻⁶⁶ we showed that ATM phosphorylates Mdmx on Ser403 in response to DSB induction, and this phosphorylation contributes to DNA damage-induced ubiquitination and degradation of Mdmx. Accordingly, the degradation of Mdmx in ATM-null cells is retarded, although not completely abolished. Since blocking PIKKs with wortmannin further blocked DSB-induced degradation of Mdmx, other members of the PIKK family might also be involved in this phosphorylation. Given the p53 paradigm, a search for additional phosphorylation events along Mdmx identified two additional serines in Mdmx that showed increased phosphorylation upon DNA damage: Ser342 and Ser367. A putative dependence of S367 and S342 phosphorylation on the PIKK kinases is under investigation. Significantly, mutating either Ser367 or Ser342 into alanine inhibited the damage-induced, Mdm2-dependent degradation of Mdmx without affecting the interaction between Mdmx and Mdm2.⁵⁷ In addition, Okamoto and coworkers showed that Ser367 phosphorylation creates a high-affinity 14-3-3 binding site (Okamoto K et al., submitted). Indeed, serine to alanine mutation of Ser367 abolished 14-3-3 binding and, as expected, the interaction between Mdmx and 14-3-3 proteins was increased upon DNA damage (Okamoto K et al., submitted). The putative role of 14-3-3/Mdmx interaction for the localization and degradation of Mdmx after DNA damage is under investigation. However, these results could indicate that the DNA damage-induced nuclear shuttling of Mdmx, which was reported to be (at least partly) independent of p53 and Mdm2, is regulated by ATM-dependent phosphorylation.⁶⁷ All in all, these data strongly suggest that the ATM-mediated DNA damage response targets Mdmx in multiple ways, resembling the regulation of p53, to ensure a tight regulation. It will be important to determine the kinases responsible for Ser367 and Ser342 phosphorylation. These results together indicate that DNA damage-activated PIKKs modulate the abundance of p53's negative regulators Mdmx and Mdm2 to ensure proper p53 activation and subsequent activation of p53-mediated damage response pathways.

The mechanism by which stability of Mdmx and Mdm2 was decreased after DNA damage remained unknown. However, we recently proposed a means for controlling the stability of Mdm2 and Mdmx upon DNA damage. While deubiquitination by HAUSP has a profound effect on the stability of both Mdm2 and p53, new evidence supports a role for HAUSP in the regulation of Mdmx stability as well.⁶⁸⁻⁷¹ The deubiquitination enzyme HAUSP was shown to directly bind and deubiquitinate Mdmx *in vitro* and *in vivo*. The expression of HAUSP is critical to maintain Mdmx protein levels under normal growth conditions. Although overexpression of HAUSP resulted in increased levels of Mdmx, increased HAUSP levels could not prevent the DNA damage-induced degradation of Mdmx. Similarly, HAUSP overexpression did not inhibit the temporary destabilization of Mdm2 upon DSB induction. Even though the intrinsic enzymatic activity of HAUSP was not decreased after DNA damage, its ability to deubiquitinate Mdmx or Mdm2 was impaired. We could show that the HAUSP-Mdmx and HAUSP-Mdm2 interactions were attenuated upon DNA damage, while the interaction between HAUSP and p53 was not diminished.⁷¹ Interestingly, and possibly expected in view of the above observations, the decreased HAUSP-Mdmx association upon DNA damage was dependent on phosphorylation of Mdmx, and could be largely restored by pretreating the cells with the PIKK-inhibitor caffeine.⁷¹ Similarly, caffeine prevented the dissociation of HAUSP and Mdm2 (Meulmeester and Jochemsen, unpublished observations). Based on the current data we propose a model in which ATM/ATR-mediated phosphorylation of Mdmx and Mdm2 results in a decreased interaction with HAUSP, rendering Mdmx and Mdm2 highly unstable upon DNA damage and unable to inhibit p53 function. On the other hand, since ATM-mediated phosphorylation activates the p53 protein, disruption of the HAUSP-Mdmx and HAUSP-Mdm2 interaction may prove to be a useful target for therapeutic intervention in the p53 pathway, to reactivate p53 in tumors with wild type p53.

CONCLUDING REMARKS

The regulation of the p53 pathway upon DNA damage is a highly regulated process. ATM orchestrates the activation of several kinases (e.g., Chk1, Chk2, c-Abl), which together impinge on different layers in the p53 pathway. ATM-dependent regulation of the abundance of p53's main negative regulators, Mdm2 and Mdmx, is emerging as an important mechanism for p53 activation. Recent data suggest that ATM/ATR regulate the interaction between HAUSP-Mdmx and HAUSP-Mdm2. We propose that this reduced interaction results in the destabilization of Mdmx and Mdm2 and allows proper p53 activation upon DNA damage. Thus, ATM directly activates p53 while activating a safe-lock mechanism to inactivate the negative regulators of p53, Mdm2 and Mdmx. The sophisticated complexity of the ATM-mediated DNA damage response ensures tight regulation of this critical arm of the cellular DNA damage response, and at the same time it fine-tunes it.

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