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Mechanism of p53 stabilization by ATM after DNA damage

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Abstract

p53 suppresses tumor development by responding to unauthorized cell proliferation, growth factor or nutrient deprivation, and DNA damage. Distinct pathways have been identified that cause p53 activation, including ARF-dependent response to oncogene activation, ribosomal protein-mediated response to abnormal rRNA synthesis, and ATM-dependent response to DNA damage. Elucidating the mechanisms of these signaling events are critical for understanding tumor suppression by p53 and development of novel cancer therapeutics. More than a decade of research has established the ATM kinase as a key molecule that activates p53 after DNA damage. Our recent study revealed that ATM phosphorylation of MDM2 is likely to be the key step in causing p53 stabilization. Upon activation by ionizing irradiation, ATM phosphorylates MDM2 on multiple sites near its RING domain. These modifications inhibit the ability of MDM2 to poly-ubiquitinate p53, thus leading to its stabilization. MDM2 phosphorylation does not inactivate its E3 ligase activity per se, since MDM2 self-ubiquitination and MDMX ubiquitination functions are retained. The selective inhibition of p53 poly-ubiquitination is accomplished through disrupting MDM2 oligomerization that may provide a scaffold for processive elongation of poly ubiquitin chains. These findings suggest a novel model of p53 activation and a general mechanism of E3 ligase regulation by phosphorylation.

Keywords

p53; MDM2; MDMX; ATM; phosphorylation; oligomerization; ubiquitination

Regulation of p53 by MDM2 and MDMX

The most notable feature of the p53 tumor suppressor is its stabilization and nuclear accumulation after exposure to many stress signals. This leads to induction of over one hundred downstream transcriptional targets that inhibit cell cycle progress, induce apoptosis, and regulate energy metabolism.[1] A critical regulator of p53 and perhaps the major factor responsible for the dynamic characteristics of p53 is the MDM2 protein. MDM2 is best known as an ubiquitin E3 ligase for p53 that promotes p53 degradation in normal cells. Although additional E3 ligases (Pirh2, Cop1) have also been shown to degrade p53 in cell culture,[2,3] mouse models of MDM2 gene knock out provided unequivocal evidence that MDM2 function is indispensable for controlling p53 activity at all stages of life.[4,5] The role of MDM2 as an important regulator of p53 stability is also validated by MDM2 knock down, and by small molecule inhibitors that disrupt p53-MDM2 binding.[6]

The MDM2 homolog MDMX is also emerging as another important regulator of p53.[7] The physiological role of MDMX was revealed by the embryonic lethality of MDMX null mice, which can be rescued by knockout of p53.[8–10] Tissue-specific knockout of MDMX generally result in mild phenotypes compared to MDM2,[5,11,12] suggesting a supplemental or

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developmental stage-specific function in p53 regulation. MDMX has weak intrinsic E3 ligase activity and does not directly promote p53 degradation.[13] Although biochemical experiments suggest that MDMX can stimulate the ability of MDM2 to ubiquitinate p53 through hetero dimerization,[14,15] the impact of MDMX expression on p53 stability is moderate at best.[16] Instead, MDMX regulates p53 mainly by formation of inactive p53-MDMX complexes.[16–18] Therefore, it appears that p53 stability is mainly regulated by MDM2.

MDM2 promotes p53 degradation by forming a stable complex through N terminal domains. After binding to p53, the MDM2 C terminal RING domain recruits ubiquitin-conjugating enzyme E2 that performs covalent modification of p53 lysine residues. In addition to E3 ligase activity, MDM2 also interacts with the proteosome subunit C8 and may deliver substrates directly to the proteasome.[19] As expected, both the p53-binding and RING domains of MDM2 are critical for p53 degradation. However, the central acidic region of MDM2 (residue 200–300) is also critical for ubiquitination of p53 through unknown mechanisms.[20,21] The acidic domain has features of a disordered region that contains binding sites for most of the MDM2-binding proteins identified to date, including transcription regulators,[22,23] de-ubiquitinating enzyme HAUSP,[24] ribosomal proteins,[25] and the tumor suppressor ARF.[26]

Stress Activation of p53

Numerous studies have established that different cellular stress and damage signals converge on MDM2 and MDMX to cause p53 activation. Oncogene-induced ARF expression induces p53 accumulation by binding MDM2 acidic domain and inhibiting p53 ubiquitination.[27] Inhibitors of rRNA transcription (such as ActD, 5-FU and growth factor deprivation) induce ribosomal stress, which stimulates MDM2 interaction with several ribosomal proteins (such as L5, L11 and L23) that also block p53 ubiquitination.[28–31] These proteins all interact with the MDM2 acidic domain, highlighting the importance of the acidic domain in sensing such growth-related stress signals.

Perhaps the most extensively studied p53 activation pathway is DNA damage signaling. A critical player in p53 DNA damage response is the ATM kinase.[32] ATM is activated within minutes after DNA double-strand break and phosphorylates numerous substrates involved in cell cycle regulation and DNA repair.[33] ATM activation of Chk2 kinase further amplifies the signal and expands the number of target proteins. Loss of ATM prevents the rapid accumulation of p53 after IR and abrogates the p53-mediated cell cycle arrest response.[32] The importance of the ATM-mediated response is not limited to direct DNA damage by irradiation. A range of physiologically-relevant stresses can also trigger ATM activation either directly or by inducing DNA damage, including chemotherapy, oncogene activation, oxidative stress, heat shock, and acidic pH.

The Role of p53 Phosphorylation in DNA Damage Response

MDM2-p53 binding has been extensively studied as a target of regulation by DNA damage. Several studies showed that DNA double-strand breaks induce phosphorylation of p53 S15 by ATM or DNA-PK.[34,35] ATM also activates Chk2, which in turn phosphorylates p53 on S20 which is part of the MDM2 binding site.[36,37] These findings suggested a model that p53 phosphorylation on the N terminus disrupts MDM2 binding and results in p53 stabilization.

However, biochemical analysis indicated that p53 S15 and S20 phosphorylation do not prevent MDM2 binding in vitro.[38] Mutation of multiple phosphorylation sites including S15 and S20 do not abrogate p53 stabilization after DNA damage in cell culture.[39–41] Mouse models showed that blocking p53 phosphorylation on S18 and S23 (equivalents of S15 and S20 in human p53) partially reduced p53 accumulation after DNA damage, and causes partial defects in apoptosis and tumor suppression.[42] S23 single site mutation also caused partial defect in p53 stabilization

by gamma irradiation and increased the incidence of B-cell lymphoma.[43] Single site mutation of S18 had no significant effect on p53 stabilization or tumor suppression, despite poor activation of certain p53 target genes after DNA damage.[44] These studies showed that p53 phosphorylation contributes to its stabilization, but also implicate the presence of additional signaling mechanisms.

The Role of MDMX Phosphorylation After DNA Damage

Befitting their roles as major regulators of p53, MDM2 and MDMX have emerged as important signaling targets by the ATM pathway. To date, the function of MDMX phosphorylation in p53 response has been extensively characterized. The results showed that ATM triggers MDMX degradation after DNA damage, eliminating its inhibitory effect on p53. We and others found that MDMX can be ubiquitinated and degraded by MDM2.[45–47] However, this reaction is tightly controlled in vivo and significantly accelerated by stress signals such as DNA damage, [46] ARF expression[47] or ribosomal stress.[48]

After DNA damage, MDMX is phosphorylated at several serine residues near the RING domain in an ATM-dependent manner (Fig. 1). Phosphorylation of MDMX led to increased binding, ubiquitination, and degradation by MDM2.[49] ATM modifies S403,[50] and Chk2 modifies S342 and S367.[49] Chk2 phosphorylation of S367 creates a binding site for 14-3-3. 14-3-3 binding promotes MDMX translocation to the nucleus by activating a cryptic NLS in the RING domain,[51] or sequesters phosphorylated MDMX in the nucleus.[52] 14-3-3 may also stimulate MDMX degradation by displacing deubiquitinating enzyme HAUSP,[52] which plays a role in MDMX degradation after DNA damage.[53,54] The physiological function of MDMX phosphorylation sites has been validated by knock-in mouse model.[55] Blocking MDMX phosphorylation in vivo dampens p53 activation after DNA damage and accelerates lymphoma development upon overexpression of c-Myc oncogene.

MDM2 Phosphorylation After DNA Damage

MDM2 phosphorylation has also been implicated in mediating p53 activation. DNA damage induces MDM2 phosphorylation on serine 395 by ATM,[56] on serine 407 by ATR,[57] and on tyrosine 394 by c-Abl.[58] Phospho-mimic mutations of these sites inhibit MDM2's ability to regulate p53 degradation or nuclear export.[56,59] The WIP1 phosphatase has been shown to inhibit p53 damage response, possibly in part by dephosphorylating MDM2 serine 395.[60] Several phosphorylation sites (targets of GSK3β) in the MDM2 acidic domain are downregulated by DNA damage,[61] and alanine substitution of S256 reduces MDM2-mediated ubiquitination of p53.[62]

Recent studies suggested two mechanisms of how MDM2 phosphorylation regulates p53 degradation. One study showed that MDM2 interaction with the scaffold protein Daxx and deubiquiting enzyme HAUSP is stimulated by DNA damage, which promote p53 de-ubiquitination.[63] A second study reported that DNA damage induces MDM2 degradation in an phosphorylation-dependent fashion,[64] suggesting that elimination of MDM2 leads to p53 stabilization. However, an earlier report showed that X-ray induces loss of MDM2 signal in western blot through ATM-dependent masking of the SMP14 epitope.[65] It is note-worthy that SMP14 was one of the MDM2 antibody used in the Stommel study. Several subsequent studies that observed MDM2 downregulation also mentioned the use of SMP14. Therefore, additional work is needed to determine whether MDM2 undergoes accelerated self-degradation after DNA damage.

Regardless of whether MDM2 undergoes a transient downregulation after DNA damage, a significant change in MDM2 activity must also occur to allow p53 stabilization. DNA damage rapidly induces MDM2 expression due to p53 activation. For at least 12 hours after DNA

damage, stabilized p53 co-exists with high level MDM2 in the cell, indicating that MDM2 has lost the ability to promote p53 degradation. In the past few years, we investigated the effect of DNA damage on MDM2 biochemical activity. The results have led to new insights on the molecular basis of this important signaling pathway, which is discussed below.

MDM2 Phosphorylation is Necessary for p53 Stabilization

In addition to the previously identified phosphorylation sites near the MDM2 RING domain (S395, S407), our mass spectrometric analysis of MDM2 from irradiated cells revealed several novel sites (S386, T419, S425 and S429). Two of these sites were confirmed to be ATM targets and were strongly induced by DNA damage.[66] The location of these sites on MDM2 show an interesting parallel to the ATM and Chk2 sites on MDMX (Fig. 1), suggesting that this region has conserved regulatory functions. Mutational analysis suggested that the MDM2 phosphorylation sites have significant redundancy in regulating p53 degradation. Phospho-mimic mutation of a single site can strongly inhibit p53 degradation. Interestingly, alanine substitution of all 6 sites resulting in MDM2-6A mutant that is hyper active in p53 degradation. Furthermore, MDM2-6A ectopic expression blocked p53 accumulation after DNA damage despite normal p53 acetylation and S15 phosphorylation.[66] These results demonstrated that MDM2 phosphorylation is critical for p53 stabilization by ATM signaling.

Because of the functional redundancy of the ATM sites on MDM2, alanine substitution of up to 3 sites showed no phenotype. From hindsight it is clear that exhaustive mutagenesis was a necessary approach, given that a single ATM site is sufficient for DNA damage response. Therefore, comprehensive mapping of all major phosphorylation sites on MDM2 was essential for evaluating their functional significance in p53 stabilization. Obviously, these findings will need to pass the ultimate test in mouse models in the future.

ATM Regulates MDM2 Oligomerization and E3 Function

How does MDM2 phosphorylation prevent degradation of p53? We found that phosphorylation inhibits the ability of MDM2 to promote p53 poly ubiquitination. Furthermore, ATM does not cause a general inhibition of MDM2 E3 ligase activity, since p53 mono ubiquitination, MDM2 self-ubiquitination and MDMX ubiquitination are not affected. Because proteasome degradation of substrates requires conjugation of poly ubiquitin chains,[67] this finding provides an attractive explanation for p53 stabilization. The results also explain a previous observation that ionizing irradiation does not eliminate low MW forms of ubiquitinated p53 (mono ubiquitination).[68]

What is the molecular basis of p53 poly ubiquitination by MDM2? Important clues can be obtained from the literature. A recent study suggests that E3 dimerization is important for promoting substrate poly ubiquitination.[69] Interestingly MDM2 RING domain produced in *E. coli* assembles into oligomers.[70] Furthermore, recombinant MDM2 C terminal fragments have a tendency to precipitate out of solution.[71] These observations suggest that MDM2 RING domain oligomerization may be key to its p53 poly ubiquitination activity, which is regulated by ATM phosphorylation.

This hypothesis was born out by our experiments. MDM2 C terminal fragment behaved as high MW oligomeric complex in gel filtration chromatography. Phosphorylation by ATM or phospho-mimic substitution inhibited oligomer formation during gel filtration, and also blocked RING domain interaction in an in vitro mixing assay.[66] These results suggest that ATM-mediated phosphorylation inhibits MDM2 RING domain homo-dimerization or oligomerization, preventing the formation of a scaffold for synthesis of poly ubiquitin chains on p53.

The structural basis of MDM2 oligomerization remains unclear. The co-crystal structure of MDM2-MDMX RING heterodimer revealed the presence of an interface that may also mediate MDM2 homo dimerization,[71] but does not indicate what structure could be responsible for higher-order interaction. Structure of BRCA1-BARD1RINGdomainheterodimershowed that sequences flanking the RING domains also engage in dimer formation.[72] Results by Poyurovsky et al. indicated that the MDM2 RING domain together with additional N and C terminal sequences (400–491) are sufficient for oligomerization.[70] The MDM2 sequence containing ATM phosphorylation sites may mediate additional interactions to stabilize the RING domain oligomer. Such interaction may be the target for regulation by phosphorylation.

How efficient is MDM2 phosphorylation by ATM in vivo? Taking advantage of phosphorylation-induced mobility shift of an MDM2 proteolytic fragment, we determined that IR induces nearly 100% phosphorylation of MDM2 even in overexpression conditions.[66] This explains the strong inhibition of RING domain oligomerization during gel filtration analysis. The observation underscores the efficiency of ATM modification, possibly attributed to the abundance of activated ATM and affinity of the MDM2 target sites. The redundancy of ATM sites may also ensure a complete shut down of the MDM2 feedback loop after DNA damage, despite MDM2 induction by p53.

MDM2 Oligomerization and p53 Poly Ubiquitination

How does MDM2 RING domain oligomerization lead to p53 poly ubiquitination? Previous studies showed that E3 ligase stimulates ubiquitination mainly by binding both substrate and E2 conjugating enzymes, bringing the reactive E2-ubiquitin thioester bond to close proximity with a substrate lysine. Nucleophilic attack by the lysine epsilon amino group results in the transfer of ubiquitin from E2 to the substrate lysine.[73–75] This “induced-proximity” model explains the substrate specificity of E3 ligases, but does not show how subsequent reactions synthesize poly ubiquitin chains necessary for substrate degradation.

To degrade p53, MDM2 must promote synthesis of poly ubiquitination chains (>4 subunits) on p53. However, p53 has 20 lysine residues and ~6–10 of these can be mono ubiquitinated by MDM2, as shown using lysine-free ubiquitin and mass spectrometry analysis. Therefore, in each round of reaction, ubiquitin can be transferred to free lysine residues on p53, or to a previously conjugated ubiquitin. Random modification of all accessible p53 lysines or random extension of all short ubiquitin chains would be inefficient for p53 degradation. Therefore, MDM2 may have a mechanism to selectively elongating a single ubiquitin chain after p53 mono ubiquitination, i.e., behaving as a processive enzyme.

Classic processive enzymes that synthesize long polymers (DNA or RNA polymerases, ribosomes) achieve un-interrupted elongation of a single product by staying engaged to the template and substrate while allowing two-dimensional sliding. This is accomplished either topologically (by the PCNA toroid during DNA replication) or through extensive but weak surface contact (between RNA polymerase and DNA during transcription).[76]

By analogy, MDM2 may gain processivity through formation of dimers and oligomers (Fig. 2). MDM2-p53 binding initiates p53 mono ubiquitination. MDM2 RING domain oligomer may favor subsequent synthesis of poly ubiquitin chain by recruiting multiple E2 molecules. It has been shown that UbcH5 family (the major E2 for MDM2) has a secondary non-covalent ubiquitin binding site.[77,78] Low-affinity ubiquitin binding by multiple E2s may attract the RING-E2 complex to the ubiquitin chain. As such, ubiquitin transfer is more likely to occur to ubiquitin instead of p53. Long ubiquitin chains will have higher affinity for RING-E3 oligomer through multiple binding, thus achieving a self-reinforcing chain elongation reaction. Previous study showed that low concentrations of MDM2 (=1 × [p53]) can only mono-ubiquitinate p53 in vitro, whereas excess MDM2 (=54 × [p53]) induces p53 poly ubiquitination.

[79] This may be due to mass-driven oligomerization of MDM2 at high concentration, conferring a poly ubiquitination activity.

After DNA damage, phosphorylated MDM2 monomers may still bind p53 and recruit activated E2, and thus can promote mono ubiquitination of p53. However, recruiting a single E2 by RING monomer may not provide sufficient affinity for the already conjugated ubiquitin, thus each round of ubiquitin transfer will occur at random with lysines on p53 or ubiquitin (Fig. 2). In addition, the termini of short ubiquitin chains that are synthesized by chance will extend out of reach by MDM2, reducing the probability of further elongation. In contrast, MDM2 oligomerization forms a large scaffold that can present E2 at various distances and angles, thus increasing the chance of chain elongation.

In addition to the model proposed above, the data do not rule out other mechanisms of poly ubiquitin chain synthesis. The secondary ubiquitin-binding site on UbcH5 allows formation of oligomers between activated UbcH5~Ubiquitin.[77] MDM2 may further stimulate and stabilize UbcH5~Ub oligomer formation and recruitment to p53. UbcH5~Ub oligomer formation may lead to pre-assembly of poly ubiquitin chain on UbcH5, allowing the poly ubiquitination of p53 through a single round of transfer. An example of such mechanism is illustrated by the gp78-Ube2g2 system.[80,81] MDM2 oligomerization may also be associated with an ability to allosterically regulate E2 activity.[82] Further experiments are needed to investigate these possibilities.

Summary and General Implications

Analysis of MDM2 phosphorylation suggests a novel mechanism of p53 regulation by ATM. The efficiency of MDM2 phosphorylation in vivo and the functional effects suggest that this may be a major mechanism of p53 stabilization after DNA damage. However, p53 stress response is likely to be achieved by multiple mechanisms acting synergistically. Phosphorylation of MDM2 also regulates interaction with HAUSP,[63] which provides an active means of de-ubiquitinating p53 and rescues the molecules from degradation. Furthermore, the p300/CBP coactivators promote p53 poly ubiquitination by acting as E4 ligase in the cytoplasm. p300/CBP encode intrinsic E3 ligase activity that can extend the ubiquitin chain initiated by MDM2.[83] Presumably, p300/CBP binding to MDM2 or p53 is needed to provide target specificity for the E4 function, which may also be regulated by DNA damage.

Targeting of proteins for proteasomal degradation requires conjugation of poly ubiquitin chain on a substrate lysine. The mechanisms of ubiquitin chain polymerization and its regulation are still under active investigation.[84] Recent studies uncovered the importance of E3 dimerization in promoting ubiquitin chain elongation. In the SCFcdc4 E3 ligase complex, dimer formation by SCF protomers is important for poly ubiquitination of substrates, whereas failure to form E3 dimer results in mono ubiquitination.[69] Defective SCF(Fbx4) dimerization in cancer prevents ubiquitination of its substrate cyclin D1 and contributes to cyclin D1 overexpression.[85] Oligomerization of the E3 ligase gp78 promotes complex formation of its cognate E2 Ube2g2 and pre-assembly of poly ubiquitin chains on the E2.[80] Formation of high-order oligomers is a common feature of RING domains.[70,86] Inhibition of RING domain self-assembly may be an effective and general mechanism for regulating E3 ligase activity. Understanding the structural basis for this regulation may provide clues for therapeutic targeting of MDM2 and other E3 ligases.

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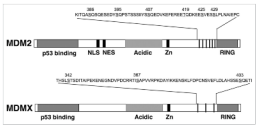

Figure 1.
Phosphorylation sites on MDM2 and MDMX. Schematic diagram shows the relative positions of phosphorylation sites near the C terminus of MDM2 and MDMX.

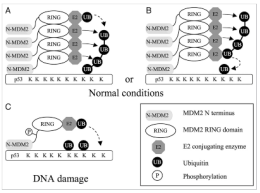

Figure 2.

A model of MDM2 regulation by phosphorylation. (a) in the absence of DNA damage, MDM2 RING domain forms oligomers that recruit multiple E2s, increasing the processivity of ubiquitin chain elongation. Ubiquitin is preferentially transferred to another proximal ubiquitin, thus building poly-ubiquitin chain on p53 through sequential transfer reactions. (B) alternatively, oligomerization of MDM2 stabilizes E2 oligomers, allowing poly-ubiquitin chain to be pre-assembled on E2 before transfering en bloc to p53. (C) after DNA damage, phosphorylation of MDM2 causes conformational changes that prevent RING domain oligomerization. Monomeric RING domain retains the ability to promote mono ubiquitination of different lysines on p53.