ATM activates p53 by regulating MDM2 oligomerization and E3 processivity

Qian Cheng¹, Lihong Chen¹, Zhenyu Li¹, William S Lane² and Jiandong Chen^{1,*}

¹Department of Molecular Oncology, Moffitt Cancer Center, Tampa, FL, USA and ²Harvard Microchemistry and Proteomics Facility, Harvard University, Cambridge, MA, USA

Rapid activation of p53 by ionizing irradiation is a classic DNA damage response mediated by the ATM kinase. However, the major signalling target and mechanism that lead to p53 stabilization are unknown. We show in this report that ATM induces p53 accumulation by phosphorylating the ubiquitin E3 ligase MDM2. Multiple ATM target sites near the MDM2 RING domain function in a redundant manner to provide robust DNA damage signalling. In the absence of DNA damage, the MDM2 RING domain forms oligomers that mediate p53 poly ubiquitination and proteasomal degradation. Phosphorylation by ATM inhibits RING domain oligomerization, specifically suppressing p53 poly ubiquitination. Blocking MDM2 phosphorylation by alanine substitution of all six phosphorylation sites results in constitutive degradation of p53 after DNA damage. These observations show that ATM controls p53 stability by regulating MDM2 RING domain oligomerization and E3 ligase processivity. Promoting or disrupting E3 oligomerization may be a general mechanism by which signalling kinases regulate ubiquitination reactions, and a potential target for therapeutic intervention.

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Introduction

The p53 pathway is functionally altered in the majority of human cancer. It is critical for maintenance of genomic stability and protection against malignant transformation. The most notable feature of p53 is its stabilization and activation after exposure to stress signals and DNA damage, which may be essential for its function as a tumour suppressor (Harris and Levine, 2005). P53 turnover is regulated by MDM2, which binds p53 and functions as an ubiquitin E3 ligase to promote p53 degradation by the proteasome (Zhang and Xiong, 2001). Mammalian cells also express an MDM2

homolog called MDMX (Shvarts *et al*, 1996). However, MDMX does not have significant intrinsic E3 ligase activity (Stad *et al*, 2001), it seems to regulate p53 mainly by formation of inactive p53–MDMX complexes (Francoz *et al*, 2006; Toledo *et al*, 2006). Therefore, it seems that p53 stability is mainly regulated by MDM2.

MDM2 promotes p53 degradation by forming a stable complex through the MDM2 and p53 N-terminal domains. On complex formation, the MDM2 C-terminal RING domain recruits ubiquitin-conjugating enzyme E2 that performs the ubiquitin covalent transfer to p53 lysine residues. Due to the importance of stable interaction between substrates and E3 ligases, p53–MDM2 binding has been extensively studied as a target of regulation by DNA damage signalling. Several landmark studies showed that DNA double strand breaks induce phosphorylation of p53 S15 by DNA-PK and ATM (Mayo *et al*, 1997; Shieh *et al*, 1997; Banin *et al*, 1998; Prives and Hall, 1999). ATM also activates Chk2, which in turn phosphorylates p53 on S20, which is a part of the MDM2-binding site (Shieh *et al*, 1997, 2000; Banin *et al*, 1998; Chehab *et al*, 2000). These findings suggested a model that p53 phosphorylation on the N terminus disrupts MDM2 binding and results in p53 stabilization.

However, biochemical analysis using phosphorylated p53 peptide indicated that S15 and S20 phosphorylation do not prevent MDM2 binding *in vitro* (Bottger *et al*, 1999). Mutation of multiple phosphorylation sites including S15 and S20 did not abrogate p53 stabilization after DNA damage in cell culture (Ashcroft *et al*, 1999; Blattner *et al*, 1999; Dumaz and Meek, 1999). Studies in mouse models showed that blocking mouse p53 phosphorylation on S18 and S23 (equivalents of human p53 S15 and S20) only partially reduced p53 stabilization after DNA damage, and causes partial defects in apoptosis and tumour suppression functions (Chao *et al*, 2006). In other reports, S23 single site mutation caused partial defect in p53 stabilization by γ irradiation (IR) and increased the incidence of B-cell lymphoma (MacPherson *et al*, 2004). Single site mutation of S18 had no significant effect on p53 stabilization or tumour suppression function, despite causing reduced C-terminal acetylation and poor activation of certain p53 target genes after DNA damage (Chao *et al*, 2003). Therefore, p53 phosphorylation clearly contributes to its stabilization after DNA damage *in vivo*. However, these results also indicate that robust p53 stabilization involves mechanisms besides p53 phosphorylation.

As the major E3 ligase for p53, MDM2 is a likely target for DNA damage signalling that leads to p53 stabilization. MDM2 is phosphorylated by ATM, ATR, and C-Abl on sites near the C terminus (Maya *et al*, 2001; Sionov *et al*, 2001; Shinozaki *et al*, 2003). However, the mechanism and significance of these modifications to p53 stabilization remain unclear. A recent study showed that MDM2 undergoes accelerated degradation after DNA damage, which may lead to p53 accumulation (Stommel and Wahl, 2004). MDM2 interaction with the scaffold protein Daxx and deubiquitinating enzyme Hausp is

*Corresponding author. Department of Molecular Oncology, Moffitt Cancer Center, 12902 Magnolia Drive, Tampa, FL 33612, USA.
Tel.: +1 813 745 6822; Fax: +1 813 745 6817;
E-mail: Jiandong.chen@moffitt.org

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stimulated by DNA damage, which may promote p53 deubiquitination and stabilization (Tang *et al*, 2006). Whether the ubiquitin E3 ligase activity of MDM2 is directly regulated by DNA damage has not been determined.

In this study, we identified multiple ATM phosphorylation sites at the C-terminal region of MDM2. These sites are phosphorylated by ATM after DNA damage, and act in a redundant manner to inhibit p53 degradation. Phospho-mimic mutation of a single site is sufficient to inhibit p53 degradation, whereas mutation of all sites is necessary for blocking p53 stabilization after DNA damage. We provide evidence that phosphorylation of MDM2 inhibits oligomerization of the RING domain and specifically blocks poly ubiquitination of p53, while having little effect on p53 mono ubiquitination. These results show that MDM2 E3 ligase function is an important target for ATM that leads to p53 stabilization and functional activation after DNA damage.

Results

Identification of novel MDM2 phosphorylation sites

To perform a comprehensive analysis of MDM2 phosphorylation status after DNA damage, 293T cells transfected with MDM2 was treated with γ IR. MDM2 was immuno purified and subjected to protease digestion and mass spectrometric analysis. The procedure achieved ~90% sequence coverage of the MDM2 protein. The results showed that in addition to previously reported S395 and S407 sites, several novel modifications sites were identified near the C-terminal RING domain, including S386, T419, S425, and S429 (Figure 1A). Four of the six phosphorylation sites were followed by glutamine (Q) that are typical targets for the ATM kinase (Kim *et al*, 1999).

To verify the mass spectrometry results and analyse the regulation of MDM2, phospho peptide-specific antibodies were raised against PS386 (a putative ATM site) and PS429 (does not conform to the ATM target motif). Both antibodies detected strong induction of MDM2 phosphorylation after γ IR of SJSA cells overexpressing MDM2 because of gene amplification (Figure 1B). The specificity of the antibodies was confirmed by lack of reactivity to non-phosphorylated His6-MDM2 purified from *Escherichia coli* (Figure 1B) or to transfected MDM2-S386A and S429A mutants after IR (Figure 1C). In addition to IR, other DNA damaging treatments including UV, etoposide, doxorubicin, and camptothecin also induced S386 and S429 phosphorylation (Figure 1D; data not shown). These results indicate that the novel C-terminal phosphorylation sites of MDM2 are highly regulated by DNA damage.

ATM-dependent phosphorylation of S386 and S429 after DNA damage

MDM2 phosphorylation was induced rapidly after IR before significant accumulation of p53, and peaked about 2.5 h in SJSA cells (Figure 2A). The rapid response is consistent with the involvement of ATM kinase. Analysis of MDM2 in ATM-deficient human skin fibroblast Hs235 showed that ATM expression was needed for S386 and S429 phosphorylation after IR (Figure 2B). Further, treatment of SJSA cells with ATM-specific inhibitor KU55933 (Figure 2C) or the less

specific ATM/ATR inhibitor caffeine (not shown) also blocked phosphorylation of S386 and S429 after IR.

To test whether S386 and S429 are direct substrates of ATM, GST-MDM2 purified from *E. coli* was incubated with ATP and FLAG-ATM immunoprecipitated from transfected 293T cells. Phosphorylation of S386 and S429 was examined by western blot using specific antibodies. The results showed that both S386 and S429 were phosphorylated by ATM *in vitro*, and the kinase-inactive ATM-KD had no effect (Figure 2D). Furthermore, Chk1 and Chk2 failed to phosphorylate S386 and S429 *in vitro*, but phosphorylated the positive control site MDMX S367 (Chen *et al*, 2005). These data indicate that ATM is responsible for directly phosphorylating S386 and S429 after DNA damage.

Mimicry of MDM2 phosphorylation specifically blocks degradation of p53

What is the functional significance of having multiple phosphorylation sites clustering near the RING domain of MDM2? Presumably, multiple sites can act in synergistic or redundant manner to regulate p53 degradation. To determine the functional significance of phosphorylation sites, MDM2 mutants with alanine, aspartic, or glutamic acid substitutions of S386 and S429 were generated. MDM2 alanine mutants remained active in degrading p53. However, S386E and S429D phospho-mimic mutants were partially defective in p53 degradation (Figure 3A). A compound MDM2 mutant (MDM2-6D) with aspartic acid substitution on all six phosphorylation sites was also defective for p53 degradation (Figure 3A). Interestingly, substitution of all six phosphorylation sites with alanine (MDM2-6A) significantly increased p53 degradation (Figure 3B and C). These results suggest that phosphorylation of a single site on MDM2 is sufficient to stabilize p53. Multiple phosphorylation sites provide redundancy in DNA damage signalling.

In addition to p53, MDMX is also a physiological substrate of MDM2 through heterodimerization with the MDM2 RING domain (Pan and Chen, 2003). To test whether the function of MDM2 RING domain is affected by the phospho-mimic substitutions, the MDM2 mutants were also tested for degradation of MDMX. The results showed that MDM2 6D and 6A mutants were fully active in the degradation of MDMX (Figure 3D). Therefore, phosphorylation by ATM does not cause a general inhibition of MDM2 E3 ligase activity. In additional assays, the MDM2-6A and MDM2-6D mutants showed normal levels of p53 and MDMX binding, and normal levels of self-ubiquitination compared with wild-type MDM2 (Supplementary Figure 1). Furthermore, the mutations did not change MDM2 half-life (data not shown).

MDM2 phosphorylation inhibits p53 poly ubiquitination

To determine how MDM2 phosphorylation inhibits p53 degradation, the ability of MDM2 mutants to promote p53 ubiquitination *in vivo* was examined by co-transfection with His6-ubiquitin. Ubiquitinated p53 was detected by western blot of Ni-NTA-captured proteins using p53 antibody. This assay preferentially detects the most abundant p53-ubiquitin species, which tends to be mono/oligo ubiquitinated p53. Although MDM2 phosphorylation-mimic mutant (MDM2-6D) was deficient in p53 degradation, it retained substantial activity in promoting p53 mono ubiquitination (Supplementary Figure 2A). However, careful examination of the

Figure 1 Identification of novel phosphorylation sites on MDM2. (A) 293T cells transiently transfected with MDM2 were treated with 10 Gy IR and 30 μ M MG132 for 4 h. MDM2 was immunoprecipitated and subjected to mass spectrometric analysis. Diagram shows MDM2 and relative positions of phosphorylation sites (underlined) identified by mass spectrometry. (B) SJSA cells were treated with 10 Gy IR and 30 μ M MG132 as indicated. MDM2 was immunoprecipitated after 4 h and probed using phosphorylation-specific antibodies. The membrane was stripped and re-probed using MDM2 antibody to confirm loading level. His6-MDM2 produced in *E. coli* was used to rule out cross reactivity. (C) 293T cells were transiently transfected with MDM2 point mutants for 48 h and irradiated with 10 Gy. MDM2 was immunoprecipitated after 4 h, probed using phosphorylation-specific antibodies, and re-probed with MDM2 antibody. (D) SJSA cells were subjected to indicated treatments (10 Gy IR, 40 J/m² UV, 70 μ M etoposide) with MG132 added to normalize MDM2 and p53 levels. MDM2 was immunoprecipitated after 4 h and probed using phospho antibodies.

banding patterns showed that MDM2-6D slightly favoured production of low MW ubiquitinated p53, whereas the MDM2-6A pattern was skewed towards higher MW (Supplementary Figure 2A).

As proteasome-mediated degradation requires poly ubiquitination (Pickart, 1997), we next performed *in vivo* ubiquitination assay using myc-ubiquitin. Western blot of p53 IP using myc antibody preferentially detects poly ubiquitinated p53 because of conjugation of multiple myc epitopes. The results showed that MDM2-6D had significantly reduced ability to induce p53 poly ubiquitination (Figure 4A, left panel). In contrast, MDM2-6A showed stronger activity in p53 poly ubiquitination than wild-type MDM2. Western blot analysis of the same samples using p53 antibody also indicated that MDM2-6A was more active in ubiquitinating p53, whereas MDM2-6D was slightly reduced in p53 mono ubiquitination (Figure 4A, right panel). Similar to the His6-ubiquitin assay, p53 antibody was not suited for detection of high molecular weight forms of poly ubiquitinated p53. MDM2-6D and MDM2-6A showed the same ability to ubiquitinate MDMX (Supplementary Figure 2B), consistent with their normal MDMX degradation function (Figure 3D). These results suggest that MDM2 phosphorylation specifically regulates p53 poly ubiquitination, with only minor effect on p53 mono ubiquitination.

To further show the effect of MDM2 phosphorylation on its E3 ligase activity, MDM2, MDM2-6A, and MDM2-6D were immunopurified from transfected H1299 cells and used to ubiquitinate p53 *in vitro*. This assay showed that MDM2-6A was more active in promoting p53 poly ubiquitination than

wild-type MDM2 *in vitro*, whereas MDM2-6D induced mainly mono-ubiquitination of p53 (Figure 4B). Wild-type MDM2 produced by transient transfection had basal phosphorylation on S386 from transfection stress (Figure 1C). As expected, dephosphorylation of wild-type MDM2 with CIP stimulated its p53 poly ubiquitination activity to a level similar to MDM2-6A. In contrast, MDM2-6A activity was not stimulated by CIP, indicating that it already has maximal activity because of lack of basal phosphorylation. MDM2-6D was weakly stimulated by CIP, presumably by dephosphorylation of other unidentified minor sites (Figure 4B). These results are consistent with the interpretation that phosphorylation of MDM2 blocks the ability to form elongated ubiquitin chains on p53, but does not prevent mono ubiquitination or short chain formation.

To further compare the effects of 6A and 6D substitutions on p53 mono ubiquitination, the *in vitro* ubiquitination assay was performed using K0-ubiquitin that only allows substrate mono ubiquitination. The result showed that MDM2 promotes p53 multi-mono ubiquitination on several lysines, resulting in products above 95 kDa. The 6A and 6D mutants showed similar activity in p53 mono ubiquitination in this assay (Figure 4C), suggesting that phosphorylation mainly inhibits the p53 poly ubiquitination function of MDM2.

DNA damage inhibits MDM2 RING domain oligomerization

Emerging evidence suggest that E3 dimerization is important for promoting ubiquitin chain extension (Tang *et al*, 2007; Barbash *et al*, 2008). Recent study showed that MDM2 RING

the C-terminal fragment (aa 362–491, Figure 5A and B) (Chen *et al.*, 1997). When analysed by size exclusion chromatography, ~50% of MDM2 RING domain purified from non-irradiated cells migrated as oligomers (~200 kDa) whereas

Figure 4 Phosphorylation of MDM2 specifically inhibits p53 poly ubiquitination. (A) P53 is co-transfected with MDM2 and myc-ubiquitin. Ubiquitinated p53 was detected by p53 IP followed by myc western blot (left panel), or p53 IP followed by p53 western blot (right panel). (B) MDM2 phosphorylation inhibits poly ubiquitination of p53 *in vitro*. MDM2 was transiently transfected into H1299 cells and captured on protein A beads using MDM2 antibody 2A9. The beads were then used to capture *in vitro* translated p53, followed by washing and incubation with E1, E2, and ubiquitin. P53 ubiquitination was detected by autofluorography. Dephosphorylation by CIP was performed on immobilized MDM2 before p53 capture. (C) MDM2 was transiently transfected into H1299 cells and captured on protein A beads using MDM2 antibody 2A9. The beads were then used to capture *in vitro* translated p53, followed by washing and incubation with E1, E2, ubiquitin, or K0-ubiquitin. P53 ubiquitination was detected by autofluorography.

the remaining 50% migrated as monomer (~15 kDa, Figure 5B). Gamma IR strongly inhibited RING oligomerization. Furthermore, inhibition of ATM with the specific inhibitor KU55933 restored RING oligomerization after IR (Hickson *et al*, 2004) (Figure 5C).

In additional experiments, the single phospho-mimic MDM2-386E mutation also caused severe defect in RING domain oligomerization (Supplementary Figure 3A), consistent with its inability to degrade p53. Furthermore, the phospho-mimic MDM2-6D RING domain produced in *E. coli* also showed reduced ability to form oligomers (Supplementary Figure 3B). These results suggest that phosphorylation inhibits MDM2 RING domain oligomerization.

Phosphorylation disrupts interaction between MDM2 RING domains

Previous study showed that contrary to convention, phosphorylated MDM2 migrates faster on SDS-PAGE (Khosravi *et al*, 1999). We were able to reproduce this observation under optimal SDS-PAGE conditions (Figure 6A, arrow). Furthermore, when the migration of 362–491 RING fragment was examined, IR treatment induced an even more dramatic change in mobility, which can be blocked by KU55933 (Figure 6B, left panel). Western blot using the PS429 antibody showed that only the fast-migrating RING fragment contains phosphorylated S429 (Figure 6B, right panel). Therefore, C-terminal phosphorylation by ATM is responsible for the fast migration of MDM2. Analysis of chromatography fractions showed that RING monomer fraction also migrated

faster than RING oligomer on SDS-PAGE (Figure 6C). Dephosphorylation by phosphatase (CIP) treatment reduced the migration rate of monomers to that of oligomers (Figure 6C). Therefore, RING oligomer is non-phosphorylated, whereas RING monomer is hyper-phosphorylated. This result suggests that phosphorylation inhibits MDM2 RING oligomerization.

To directly test whether phosphorylation inhibits MDM2 RING domain interaction, full-length MDM2 was incubated with a mixture of phosphorylated and non-phosphorylated RING domain to determine which form is preferentially pulled down. The results showed that the non-phosphorylated RING bound to MDM2 with much higher affinity than phosphorylated RING (Figure 6D and E). Overall, these results suggest that ATM-mediated phosphorylation inhibits MDM2 RING domain homo-dimerization or oligomerization, preventing the formation of a scaffold for processive ubiquitin chain elongation.

Phosphorylation-resistant MDM2 prevents p53 stabilization after DNA damage

To determine the functional significance of MDM2 phosphorylation in p53 response to DNA damage, U2OS cell lines with stable expression of ectopic MDM2 were established. MDM2-6A was expressed as a physiologically relevant level similar to SJSA cells (Supplementary Figure 4A). IR induced p53 accumulation after 1 h in control U2OS cells (Figure 7A). Ectopic expression of wild-type MDM2 did not prevent p53 stabilization, suggesting that DNA damage signals can neutralize the

Figure 5 DNA damage inhibits MDM2 RING domain oligomerization. (A) Caspase 3 cleaves MDM2 at a single site (361) and releases the C-terminal fragment 362–491 containing the phosphorylation sites and RING domain. (B) SJSA lysate was incubated with recombinant Caspase 3 and analysed by western blot with MDM2 C-terminal antibody 4B11. (C) SJSA cells were treated with 10 Gy IR in the absence or presence of KU55933. MDM2 was immunoprecipitated with 2A9 after 2 h, cleaved with Caspase 3, and the C-terminal fragment (362–491) was analysed by gel filtration chromatography and western blot using 4B11 antibody. Note that the RING fragment migrates on SDS–PAGE as a ~30 kDa band, which is slower than its calculated molecular weight of 15 kDa.

Figure 6 Phosphorylation alters MDM2 mobility and inhibits RING domain interaction. (A) SJSA cells were treated with 10 Gy IR in the presence of MG132. MDM2 was detected by 3G9 western blot. Arrow indicates faster migration of a subpopulation of MDM2. (B) SJSA cells were treated with 10 Gy IR in the presence of MG132 and KU55933 for 2 h. MDM2 was cleaved with Caspase 3 and analysed by 4B11 (left panel) and PS429 (right panel) western blot. (C) SJSA MDM2 was cleaved with Caspase 3 and fractionated by gel filtration chromatography. Oligomer (early) and monomer (late) column fractions were treated with CIP and analysed for mobility change by SDS–PAGE. The result indicates that RING monomer is hyper-phosphorylated. (D) MDM2 immunoprecipitated from untreated and irradiated SJSA cells was cleaved by Caspase 3 and analysed by Western blot with 4B11 (left panel) and PS429 (right panel) antibody. (E) Quantitation of result in (D) by densitometry.

Figure 7 Inhibition of p53 stabilization by MDM2 phosphorylation mutant. **(A)** U2OS cells stably transfected with wild type and MDM2-6A were treated with 10 Gy IR and analysed at indicated time points for induction of p53 by western blot. **(B)** U2OS cells stably transfected with MDM2-6A was treated with DNA damaging drug neocarzinostatin and analysed by western blot for p53 stabilization. **(C)** U2OS cells stably transfected with wild type and MDM2-6A were treated with indicated agents for 8 h and analysed for induction of p53 by western blot.

effect of high level MDM2. However, MDM2-6A expression blocked p53 accumulation for up to 8 h after IR (Figure 7A), similar to the delay reported earlier in ATM-deficient fibroblasts. The effect likely required mutation of all six phosphorylation sites, because MDM2 mutants with single alanine substitution (386A or 429A) and the MDM2-3A mutant (386A/395A/429A) did not prevent p53 accumulation (Supplementary Figure 4B). Failure to accumulate p53 was associated with significant defect in p21 induction (Figure 7A). MDM2-6A also blocked p53 activation by persistent DNA damage in the presence of neocarzinostatin (Figure 7B) or etoposide (Supplementary Figure 4C), but did not affect actinomycin D (ribosomal stress), 5-FU (ribosomal stress), and Nutlin (p53–MDM2 disruptor) (Figure 7C). These results indicate that MDM2 phosphorylation is specifically required for p53 activation after DNA damage.

DNA damage also stimulates p53 phosphorylation and acetylation that are implicated in its functional activation. U2OS cells expressing MDM2-6A were severely defective in p21 induction after DNA damage. However, p53 S15 phosphorylation and K382 acetylation levels (the ratio of modified versus total p53) were not inhibited by MDM2-6A after IR (Supplementary Figure 4D). This result suggests that in the absence of protein accumulation, p53 phosphorylation and acetylation are not sufficient for its functional activation.

Phosphorylation-resistant MDM2 interferes with p53 function

The phenotype of MDM2-6A provided an opportunity to test the biological consequence of preventing p53 accumulation after DNA damage. U2OS cells expressing MDM2 mutants

were treated with 10 Gy IR and analysed by ^3H -thymidine incorporation assay. The results showed that MDM2-6A expression partially abrogated the ability to undergo cell-cycle arrest after DNA damage (Figure 8A). This is expected as MDM2-6A prevents induction of p21 after IR.

To further test the effect on long-term cell survival, cells were treated with 0–7.5 Gy IR and colony formation efficiency was determined after culturing for 15 days. MDM2-6A expression significantly decreased long-term cell viability after IR (Figure 8B), suggesting that rapid stabilization of p53 is critical for promoting long-term survival, possibly by inducing cell-cycle arrest and allowing timely repair of damage. Suppression of p53 expression by stable shRNA retrovirus vector also resulted in the same phenotype (Supplementary Figure 5). This is consistent with previous finding that p53 knockout in HCT116 cells enhanced sensitivity to DNA damage (Bunz *et al*, 1999).

Discussion

Mouse models clearly showed a role for p53 N-terminal phosphorylation sites in p53 stabilization, but also indicated significant contribution from additional mechanisms. Our data suggest that ATM phosphorylation of MDM2 is important for rapid stabilization of p53 by DNA damage. We showed that MDM2 phosphorylation inactivates its ability to poly ubiquitinate p53, possibly by preventing oligomerization of MDM2 RING domain. The results also showed that when p53 accumulation is blocked by ATM-resistant MDM2-6A, p53 target gene expression is not induced after DNA damage despite normal phosphorylation and acetylation of

Figure 8 Regulation of cell cycle and long-term survival after IR by MDM2 mutants. (A) U2OS cells stably expressing Wt MDM2 and MDM2-6A were treated with 10 Gy IR and DNA synthesis was determined by ^3H -thymidine incorporation assay after 8 and 24 h. (B) U2OS stably expressing wild type and mutant MDM2 were plated at low density, treated with IR, cultured for 15 days, and stained for colony formation.

Figure 9 A model of MDM2 regulation by phosphorylation. In the absence of DNA damage, MDM2 RING domain forms oligomers that recruit multiple E2s, increasing the processivity of ubiquitin chain elongation. After DNA damage, phosphorylation of MDM2 causes conformational changes that prevent RING domain oligomerization. Monomeric RING domains catalyze ubiquitination in a dissociative manner, resulting in mono ubiquitination of different lysines on p53.

p53. Therefore, p53 accumulation is necessary for robust functional activation by DNA damage.

Our data suggest that multiple phosphorylation sites on MDM2 act in a redundant manner to mark an important signalling target for p53 activation. Phosphorylation-mimic substitution of a single site strongly inhibits p53 degradation, whereas alanine substitution of all six sites is needed to prevent signalling to p53. This finding explains the observation that DNA damage signalling can stabilize p53 despite induction of MDM2 expression. Furthermore, cells with MDM2 amplification or constitutive ectopic MDM2 overexpression still retain significant p53 stabilization after DNA damage. Multiple ATM sites on MDM2 may be responsible for ensuring a temporary shutdown of the negative feedback loop, as indicated by the complete phosphorylation (mobility shift) of the entire MDM2 population after IR (Figure 6B). DNA damage has been shown to induce rapid degradation of MDM2 partly through the ATM pathway (Stommel and Wahl, 2004). However, mutating all six MDM2 phosphorylation sites did not change MDM2 stability. In related experiments, we did not observe convincing changes in wild-type MDM2 stability or steady-state level before and after IR treatment (unpublished observations). Instead, our results suggest that

C-terminal phosphorylation regulates the ability of MDM2 to promote p53 poly ubiquitination, with only minor effects on p53 mono ubiquitination.

Our results are consistent with the model (Figure 9) that in the absence of stress, MDM2 RING domain forms dimer or oligomers that recruit multiple E2s. This process increases the processivity of ubiquitin chain elongation reaction, resulting in p53 poly ubiquitination and degradation. After DNA damage, phosphorylation near the RING domain by ATM causes conformational change or steric interference that prevents RING oligomerization. The monomeric RING domain can mono ubiquitinate p53 on different lysine residues, but has no preference for chain elongation. Efficient targeting of substrates to the proteasome requires formation of linear ubiquitin chains containing at least four subunits (Thrower *et al*, 2000). Therefore, p53 mono ubiquitination will not lead to degradation, and may even facilitate its mitochondrial targeting and functional activation (Le Cam *et al*, 2006; Marchenko *et al*, 2007). P53 dimerization is also important for ubiquitination by MDM2 possibly by facilitating stable binding to MDM2 (Kubbutat *et al*, 1998; Maki, 1999), which is another key requirement for processive chain elongation.

Targeting of proteins for proteasomal degradation requires conjugation of ubiquitin to a substrate lysine, followed by elongation of K48-linked poly ubiquitin chain. Much has been learned about the regulation of substrate-binding step needed for initial ubiquitination of a substrate lysine. However, the mechanism of ubiquitin chain polymerization and its regulation is still under active investigation. Recent studies begin to show the importance of E3 dimerization in promoting ubiquitin chain elongation. In the multimeric SCF^{Cdc4} E3 ligase complex, dimer formation by SCF proto-mers is important for poly ubiquitination of substrates, whereas failure to form E3 dimer results in substrate mono ubiquitination (Tang *et al*, 2007). Lack of SCF(Fbx4) dimerization in cancer prevents ubiquitination of its substrate cyclin D1, which contributes to cyclin D1 overexpression and transformation (Barbash *et al*, 2008).

Mechanistically, formation of high-order structures by E3 helps to present multiple E2 active sites to the substrate and increase the reaction rate. Oligomerization may facilitate stable binding and processive elongation of ubiquitin chain using secondary ubiquitin-binding sites on the E2 (Brzovic *et al*, 2006). Oligomerization of E3 may also increase access to lysines on the elongating ubiquitin chain because of availability of charged E2s at different orientations. Formation of high-order oligomers is a common feature of RING domains (Kentsis *et al*, 2002; Poyurovsky *et al*, 2007). Oligomerization of MDM2 RING domain increases its E3 ligase activity *in vitro* (Poyurovsky *et al*, 2007). Results described in this report showed that inhibition of RING domain self-assembly is a novel mechanism of MDM2 regulation by ATM. It is possible that similar mechanism may be used in the regulation of other E3s.

Materials and methods

Cell lines and plasmids

The ATM-deficient Hs235 cell line was purchased from the ATCC. Human foreskin fibroblast was provided by Dr Jack Pledger. FLAG-ATM expression plasmids were provided by Dr Michael Kastan. MDM2 point mutants were generated by site-directed mutagenesis using the QuickChange kit (Stratagene). All MDM2 and MDMX constructs used in this study were human cDNA clones. MDM2 inhibitor Nutlin (Cayman) was used at 5–10 μ M. Neocarzinostatin was provided by the NIH. U2OS cells with stable expression of MDM2 mutants were generated by transfection of CMV-driven MDM2 plasmids followed by G418 selection and isolation of clonal cell lines.

Protein analysis

To detect proteins by western blot, cells were lysed in lysis buffer [50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% NP40, 1 mM PMSF, 50 mM NaF] and centrifuged for 5 min at 10 000 g. Cell lysate (10–50 μ g protein) was fractionated by SDS-PAGE using gradient gel and transferred to Immobilon P filters (Millipore). The filter was blocked for 1 h with phosphate-buffered saline containing 5% non-fat dry milk, 0.1% Tween-20. The following monoclonal antibodies were used: 4B2 and 2A9 for full-length MDM2 IP (Chen *et al*, 1993), 3G9 for western blot of full-length MDM2, and 4B11 for western blot of MDM2 C-terminal fragment, DO-1 (Pharmingen) for p53 western blot, 8C6 antibody for MDMX western blot and IP (Li *et al*, 2002). The filter was developed using ECL-plus reagent (Amersham). Phosphorylation-specific rabbit antibodies were raised against phosphorylated MDM2 peptides TQA(pS)QSQES (PS386) and VES(pS)LPLNA (PS429) (GenScript) and affinity purified. MDM2 phosphorylation analysis was carried out by immunoprecipitation using 2A9 antibody from 0.5–2 mg cell extract followed by western blot using the phosphorylation-specific antibodies.

Affinity purification of MDM2 and phosphorylation site mapping

Transformed human embryonic kidney 293T cells were transfected with MDM2 expression plasmid for 48 h and treated with 10 Gy γ IR and 30 μ M MG132 for 4 h. Cells ($\sim 2 \times 10^8$) were lysed in 10 ml lysis buffer [50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% NP40, 1 mM PMSF, 200 mM Okadaic acid]. The lysate was precleared with 100 μ l bed volume of protein A sepharose beads for 30 min, and then incubated with 50 μ l protein A sepharose beads and $\sim 10 \mu$ g 2A9 antibody for 4 h at 4°C. The beads were washed and MDM2 was eluted by boiling in Lammilae sample buffer, fractionated on SDS-PAGE, and stained with Coomassie Blue. Gel slice containing the MDM2 band was subjected to mass spectrometric analysis at the Harvard Microchemistry Facility as described earlier (Chen *et al*, 2005).

In vitro kinase reactions

For each phosphorylation reaction *in vitro*, 5×10^7 293T cells transiently transfected with 20 μ g FLAG-ATM expression plasmid were immunoprecipitated using M2-agarose beads as described earlier (Kim *et al*, 1999). GST-MDM2-294–491, GST-MDMX-280–490 (200 ng) purified from *E. coli* were incubated with M2 beads containing FLAG-ATM or FLAG-Chk2 in kinase buffer (20 mM HEPES pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT, and 10 mM MnCl₂) with 5 mM ATP for 1 h at 30°C. The samples were boiled and fractionated on SDS-PAGE and subjected to western blot analysis using MDM2 and MDMX phosphorylation-specific antibodies.

In vivo ubiquitination assay

H1299 cells in 10 cm plates were transfected with 5 μ g His6-ubiquitin, 5 μ g MDMX, 1–2 μ g MDM2, and 1 μ g p53 expression plasmids using calcium phosphate precipitation method. Thirty-two hours after transfection, cells from each plate were collected into two aliquots. One aliquot (10%) was used for direct western blot. The remaining cells (90%) were used for purification of His6-tagged proteins by Ni²⁺-NTA beads. The cell pellet was lysed in buffer A (6 M guanidinium-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄, 0.01 M Tris-Cl pH8.0, 5 mM imidazole, 10 mM β -mercaptoethanol) and incubated with Ni²⁺-NTA beads β (Qiagen) for 4 h at room temperature. The beads were washed with buffer A, B (8 M urea, 0.1 M Na₂PO₄/NaH₂PO₄, 0.01 M Tris-Cl pH8.0, 10 mM β -mercaptoethanol), C (8 M urea, 0.1 M Na₂PO₄/NaH₂PO₄, 0.01 M Tris-Cl pH6.3, 10 mM β -mercaptoethanol), and bound proteins were eluted with buffer D (200 mM imidazole, 0.15 M Tris-Cl pH6.7, 30% glycerol, 0.72 M β -mercaptoethanol, 5% SDS). The eluted proteins were analysed by western blot for the presence of conjugated p53 or MDMX. Alternatively, myc-ubiquitin was used in place of His6-ubiquitin. Cells were treated with MG132 for 4 h and precipitated using p53 or MDMX antibodies in the presence of 10 mM iodoacetamide, and probed with anti-myc epitope antibody by western blot.

In vitro ubiquitination assay

MDM2 plasmid (10 μ g) was transfected into H1299 cells for 48 h, treated with 30 μ M MG132 for 4 h, and immunoprecipitated with 2A9 antibody. The substrate p53 was produced by *in vitro* translation in rabbit reticulocyte lysate using the TNT system (Promega) in the presence of ³⁵S-methionine; 15 μ l packed protein A beads loaded with MDM2 from a 10 cm plate of H1299 cells were treated with 1 U CIP for 0.5 h at 37°C when indicated, washed with lysis buffer and reaction buffer (50 mM Tris pH7.5, 2.5 mM MgCl₂, 15 mM KCl, 1 mM DTT, 0.01% Triton X-100, 1% glycerol), incubated with 10 μ l of *in vitro* translated p53 for 2 h at 4°C, and washed with reaction buffer. The beads loaded with MDM2–p53 complex were incubated with 250 ng purified human ubiquitin-activating enzyme His6-E1, 100 ng His6-Ubc5Hb ubiquitin-conjugating enzyme, 5 μ g ubiquitin (BIOMOL), 5 μ g K0-ubiquitin (Boston Biochem), and 20 μ l reaction buffer in the presence of 4 mM ATP. The mixture was incubated at 37°C for 2 h with shaking, boiled in SDS sample buffer, and fractionated by SDS-PAGE. The gel was dried and p53 was detected by autoradiography.

Gel filtration chromatography

Bacterial lysate expressing GST-MDM2 and GST-MDM2-6D were applied to glutathione-agarose beads according to the manufacturer's protocol (Pierce). The bound GST-MDM2 proteins were

cleaved by purified His6-Capase 3 at 37°C for 1 h to release C-terminal fragment (362–491) (Chen *et al*, 1997). For analysis of endogenous MDM2, five 15 cm plates of SJSA cells were treated with 8 µM Nutlin for 16 h to increase MDM2 level, irradiated with 10 Gy IR for 1 h, immunoprecipitated using MDM2 antibody 4B2, and cleaved with Caspase 3. The cleaved MDM2 fragments were applied to a Superdex 200 Hiload 16/60 column and fractionated on an AKTA Purifier chromatography system (Amersham Biosciences). The column was equilibrated and eluted with buffer containing 20 mM Hepes pH 7.5 and 400 mM NaCl. The eluted fractions were subjected to western blot using 4B11 antibody against the RING domain. The sizes of RING fragment complexes were determined by comparing the elution profile to a molecular weight standard fractionated under identical conditions.

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Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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