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# SUMO-1 Modification of Mdm2 Prevents Its Self-Ubiquitination and Increases Mdm2 Ability to Ubiquitinate p53

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## Summary

**Mdm2 is an E3 ubiquitin ligase for the p53 tumor suppressor protein. We demonstrate that Mdm2 is conjugated with SUMO-1 (sumoylated) at Lys-446, which is located within the RING finger domain and plays a critical role in Mdm2 self-ubiquitination. Whereas mutant $Mdm2^{K446R}$ is stabilized, it elicits increased degradation of p53 and concomitant inhibition of p53-mediated apoptosis. In vitro sumoylation of Mdm2 abrogates its self-ubiquitination and increases its ubiquitin ligase activity toward p53. Radiation caused a dose- and time-dependent decrease in the degree of Mdm2 SUMO-1 modification, which is inversely correlated with the levels of p53. Our results suggest that the maintenance of the intrinsic activity of a RING finger E3 ubiquitin ligase is sumoylation dependent and that reduced Mdm2 sumoylation in response to DNA damage contributes to p53 stability.**

## Introduction

Proteolysis plays an important role in regulating the cell's proliferation, differentiation, and response to stress. A delicate balance between protein protection from or targeting for degradation underlies the regulation of proteolysis and determines the duration and magnitude of activities elicited by key regulatory proteins. Covalent attachment of polyubiquitin is required for the efficient degradation of proteins by the 26S proteasome complex (Hershko and Ciechanover, 1998). Conjugation of the ubiquitin polypeptide is mediated by multiple enzymatic reactions catalyzed by a single ubiquitin-activating enzyme (E1), a few ubiquitin-conjugating enzymes (E2s), and a large variety of ubiquitin ligases (E3s). Whereas recognition of substrates by E3 ubiquitin ligases dictates the specificity of protein ubiquitination, the intrinsic ubiquitin ligase activity represents a rate-limiting step of ubiquitin conjugation. Therefore, the mechanisms underlying the regulation of E3 are central to the control of proteasome-dependent protein degradation in vivo.

Two major classes of E3 ubiquitin ligases are known. The first is represented by HECT (homologous to E6AP carboxy terminus) ligases, in which the formation of ubiquitin-thioester intermediates is followed by a direct transfer of ubiquitin onto a substrate (Nuber and Scheffner, 1999), as shown for E6AP, RSP5, and Nedd4 (Huibregtse et al., 1995; Schwarz et al., 1998). The other E3 class, whose activity depends on the presence of a RING finger motif, does not form thioester intermediates with ubiquitin (Chen et al., 2000). Small RING finger protein ROC1/Rbx1/Hrt1 mediates ubiquitin ligation within the multiprotein E3 complexes, such as Skp1-Cullin1-F box protein-ROC1 (SCF-ROC1) and VHL-elongin B-elongin C-Cullin2-Roc1 (VCB-Cul2-ROC1) (Kamura et al., 1999; Tan et al., 1999; Wu et al., 2000). These E3s mediate ubiquitination of cyclin-dependent kinase inhibitors, yeast cyclins, inhibitor of NF-κB transcription factor (IκB), β-catenin, and α subunit of hypoxia-inducible factor (reviewed in Deshaies, 1999). APC11, a ROC1 homolog, participates in the formation of the anaphase-promoting complex (APC) ubiquitin ligase and mediates ubiquitination of cyclins A and B, Pds1, and Geminin (reviewed in Zachariae and Nasmyth, 1999).

Whereas ROC1 and APC11 are part of a multiprotein complex and rely on their partners (i.e., F box proteins) for substrate recognition, recent studies reveal a rapidly growing number of RING finger proteins, which directly bind their substrate and exhibit ubiquitin ligase activity. Among those are mammalian homologs of *seven in absentia*, AO7, cCbl/Sli-1, BRCA1, and Mdm2 (Hu et al., 1997; Honda and Yasuda, 1999; Levkowitz et al., 1999; Lorick et al., 1999).

As an oncogene, Mdm2's transforming potential is largely attributed to its ability to downregulate the functions of the p53 tumor suppressor protein. The disruption of p53 biological activities often seen in human tumors is, at least in part, attributed to Mdm2 overexpression. Association of Mdm2 with p53 abrogates p53 transcriptional activities (Chen et al., 1995). Moreover, Mdm2 has been implicated in the proteasome-dependent degradation of p53 (Haupt et al., 1997; Kubbutat et al., 1997). Mdm2 has been shown to target p53 ubiquitination in vitro and in vivo (Honda et al., 1997; Fuchs et al., 1998). Although initially believed to form the ubiquitin-thioester intermediate (Honda et al., 1997), Mdm2 does not contain the HECT domain. Recent evidence suggests that Mdm2 mediates p53 ubiquitination via the carboxyl terminal RING finger domain and that Mdm2 is a bona fide RING finger E3 ubiquitin ligase (Fang et al., 2000; Honda and Yasuda, 2000).

The ubiquitination of p53 by Mdm2 is tightly regulated in mammalian cells. Mdm2 effects on p53 are attributed to the abundance of Mdm2 expression, the levels of Mdm2-p53 association, and modulation of Mdm2 ubiquitin ligase activity. Mdm2 levels are regulated by its substrate p53, which activates *Mdm2* gene transcription (Barak et al., 1993). Stabilization of p53 in response to stress and DNA damage is due to Mdm2 dissociation from phosphorylated p53 (Shieh et al., 1997; Siliciano et al., 1997; Fuchs et al., 1998). The accumulation of

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p53 may be also attributed to the inhibition of Mdm2 ubiquitin ligase activity upon its association with $p14^{ARF}$ or MdmX (Kamijo et al., 1998; Honda and Yasuda, 1999; Sharp et al., 1999).

Mutations of conserved cysteine residues or deletions in the Mdm2 RING finger domain abolish its ability to target p53 ubiquitination and prolong Mdm2's half-life (Fang et al., 2000; Honda and Yasuda, 2000). Since Mdm2 is capable of self-ubiquitination (Honda and Yasuda, 1999) and is degraded by the proteasome pathway with kinetics that are similar to those of p53, it is unclear how the intrinsic ubiquitin ligase activity of Mdm2 may be preserved during p53 ubiquitination. Such a mechanism would be expected to distinguish between Mdm2 self-ubiquitination and the ubiquitination of its NH2-terminal associated protein, p53.

The sidechains of lysil residues serve as a conjugation site for ubiquitin, as well as for the small ubiquitin-like modifier protein SUMO-1 (Johnson et al., 1997; Kamitani et al., 1997). Covalent attachment of SUMO-1 (also termed Sentrin, Ubl1, or, in yeast, Smt3) to lysines was demonstrated for the RanGAP1 (Ran GTPase activating protein1) (Mahajan et al., 1997), IκBα (Desterro et al., 1998), PML (Kamitani et al., 1998), and p53 (Gostissa et al., 1999; Rodriguez et al., 1999). SUMO-1, which is 18% identical to ubiquitin, utilizes a similar conjugation pathway consisting of activation by a heterodimeric Aos1-Uba2 activating enzyme (E1) and conjugation onto a substrate by Ubc9 (Johnson and Blobel, 1997; Johnson et al., 1997), a protein with a strong sequence similarity to ubiquitin-conjugating enzymes (E2s). Unlike ubiquitin, SUMO-1 does not conjugate to itself and thus is not capable of forming chains similar to the polyubiquitin, which are required for recognition by the 26S proteasome.

Although SUMO-1 is an essential gene in *Saccharomyces cerevisiae*, the function of SUMO-1 modification (sumoylation) is not well understood. SUMO-1 conjugation is implicated in altering the subcellular localization of its RanGAP1, Sp100, and PML substrates (Mahajan et al., 1997; Duprez et al., 1999; Strensdorf et al., 1999), in regulating septin ring dynamics during the cell cycle in budding yeast (Johnson and Blobel, 1999), and in neural differentiation in *Drosophila* (Lehembre et al., 2000). Sumoylation of p53 is believed to increase its transcriptional activities (Gostissa, et al., 1999; Rodriguez et al., 1999). SUMO-1 has an entirely different effect on IκBα, where it becomes attached to the major ubiquitination site (Lysine 21; Desterro et al., 1998), thereby preventing ubiquitination and protecting sumoylated IκBα from proteasome-dependent proteolysis.

RING finger domains in E3 ubiquitin ligases were demonstrated to recruit E2 ubiquitin-conjugating enzymes (Lorick et al., 1999; Chen et al., 2000). The facts that Ubc9 is highly homologous to ubiquitin-conjugating enzymes, that Mdm2 binding partner p53 is sumoylated, and that one of the critical sumoylation sites of PML is located within its RING finger domain prompted us to examine the possibility that Mdm2 serves as a substrate for SUMO-1 conjugation. Here, we demonstrate that Mdm2 is sumoylated and that Mdm2 stability and ligase activity are altered as a consequence of SUMO-1 conjugation.

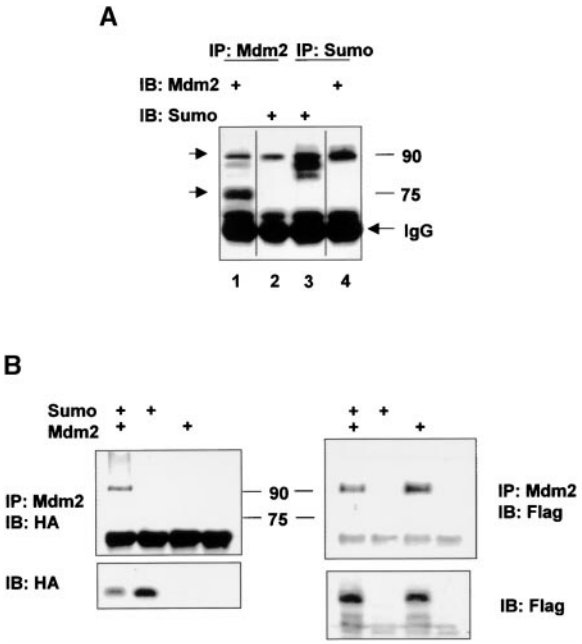


Figure 1. SUMO-1 Is Conjugated to Mdm2 In Vivo

(A) Whole cell extracts (WCE) prepared from normal human fibroblasts were immunoprecipated (IP; 500 μg) with monoclonal antibodies either to Mdm2 or to SUMO-1 and analyzed by immunoblotting with the indicated antibodies.

(B) 293T cells were transfected with Flag-Mdm2 and HA-SUMO-1. WCE prepared 24 hr later were immunoprecipitated using monoclonal antibodies against Mdm2 and analyzed by immunoblotting using monoclonal antibodies to HA or to Flag as indicated in the figure. Right part of the figure depicts the expression level of the transfected constructs.

## Results

### Mdm2 Is Conjugated with SUMO-1 in Human Cells

Analysis of Mdm2 expression by immunoblotting often reveals multiple forms that are detected by different Mdm2 antibodies. To test whether some of these forms could result from SUMO-1 modification of Mdm2, we monitored the possible presence of SUMO-1-conjugated Mdm2 in vivo. For this purpose, we immunoprecipitated Mdm2 or SUMO-1 from denatured normal human fibroblast cell lysates (to inhibit the activity of desumoylation enzymes) and analyzed the samples by immunoblotting with the monoclonal antibodies against Mdm2 and SUMO-1. Of the two major Mdm2-antibody-reacting bands (with apparent molecular weight of 75 and 90 kDa; Figure 1A, lane 1), only 90 kDa protein was recognized by SUMO-1 antibody (Figure 1A, lane 4). SUMO-1 conjugation with Mdm2 was confirmed by a reciprocal experiment in which proteins were first immunoprecipitated with Mdm2 antibody and analyzed in Western blot with the SUMO-1 antibody (Figure 1A, lane 2). These data demonstrate that endogenous Mdm2 with an apparent molecular weight of 90 kDa is conjugated with SUMO-1. The change in mobility of sumoylated Mdm2 revealed an ~15 kDa shift, which suggests that Mdm2 modification can be attributed to the conjugation of a single SUMO-1 molecule.

To confirm this finding, we transfected 293T cells with

Flag-tagged Mdm2 and HA-tagged SUMO-1 constructs. In these cells, we found that all detectable exogenous Mdm2 was of ~90 kDa (Figure 1B, right panel). Analysis of Mdm2 immunoprecipitates by HA antibody clearly showed that Mdm2 is conjugated with HA-SUMO-1 (Figure 1B, left panel).

### Lys-446 Is Required for Mdm2 Sumoylation

The major lysine residue for SUMO-1 conjugation in PML protein is Lys-65, the fifth residue distal to the beginning of the first loop of the RING finger (Kamitani et al., 1998). Comparison of the RING finger motifs of PML and Mdm2 revealed the presence of a similarly distanced Lys-446 within the RING finger of Mdm2. Analogously located lysines are found in other RING finger proteins, including murine Mdm2 (K444), equine Mdm2 (K446), BRCA1 (K32), Praja1 (K359), and AO7 (K144).

To determine which lysine residues within the RING finger are required for the sumoylation of Mdm2, we mutated the respective amino acids, generating three mutants: K1 (K446R), K2 (K453R), and K3 (K465,466, 468,469,472R). Coexpression of HA-SUMO-1 and the lysine-mutated forms of Mdm2 revealed that K2 and K3 exhibit the same mobility as the wild-type (wt) form of Mdm2, although expressed to somewhat lower levels. Conversely, the K1 mutant of Mdm2 migrated as a 75 kDa protein (Figure 2A, middle panel). The latter resembles one of the endogenously expressed Mdm2 species (Figure 1A) and may represent a nonsumoylated form of Mdm2. These data imply that the exogenously expressed wt Mdm2 is efficiently sumoylated, whereas the K1 mutant is not. Furthermore, analysis of Flag immunoprecipitates by immunoblotting with HA antibody revealed that the K1 mutant is not conjugated with SUMO-1 in vivo (Figure 2A, upper panel). Thus, Lys-446 is the major sumoylation site on Mdm2. These findings also demonstrate that mutations on other lysines within the RING finger domain do not significantly impair Mdm2 sumoylation, although we cannot exclude the possibility that residual sumoylation may occur at the lysine residues outside of the RING finger.

We directly assessed the sumoylation of Mdm2 in vitro. Bacterially expressed wt or mutant forms of Mdm2 were incubated with purified Aos1/Uba2, Ubc9, and in vitro translated immunopurified $^{35}$S-labeled SUMO-1 followed by SDS-PAGE and autoradiography (Figure 2B, upper panel) or Coomassie Blue staining (Figure 2B, lower panel). As evident from both the $^{35}$S-detected signal and the mobility of Mdm2 proteins, SUMO-1 was conjugated with the wt Mdm2, as well as with the K2 and the K3 forms, but not with the K1 mutant. These data provide direct evidence for Mdm2 sumoylation at Lys-446, which is similar to one of the major PML sumoylation sites.

### Sumoylation of Mdm2 Inhibits Its Self-Ubiquitination

To investigate whether SUMO-1 and ubiquitin utilize a similar conjugation site, we carried out an in vitro ubiquitination reaction of the wt or K1 mutant forms of Mdm2. Incubation of Mdm2 proteins with E1, E2 (UbcH5b), $^{32}$P-ubiquitin, and ATP resulted in detectable ubiquitination of wt Mdm2 but not of the K1 Mdm2 mutant (Figure 3A, lane 3 versus lane 4). This result demonstrates that Lys-446 serves as a major site for Mdm2 self-ubiquitination, although we cannot rule out residual ubiquitination of other lysines within Mdm2 and/or GST moieties. Together with the data presented in Figure 2, our findings suggest that SUMO-1 may compete with ubiquitin for conjugation at Lys-446.

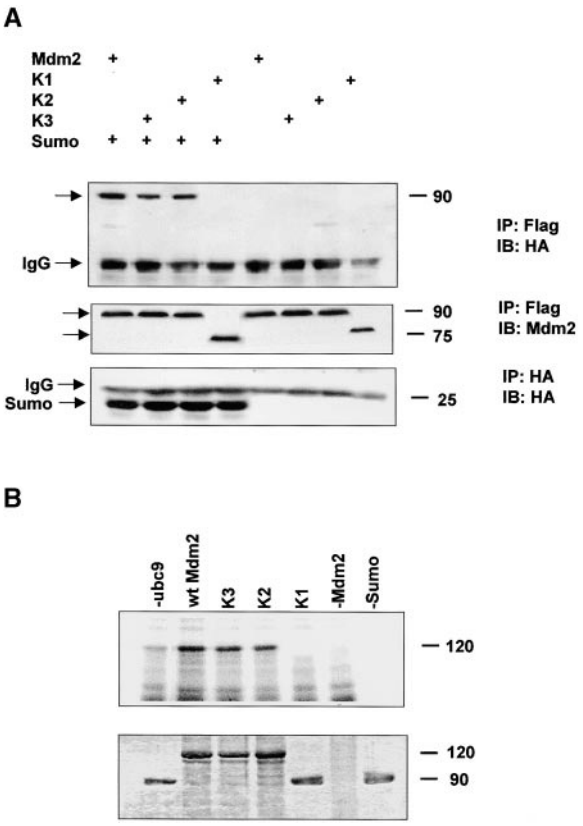


Figure 2. Mutation of Lys-446 in Mdm2 Abolishes SUMO-1 Conjugation

(A) 293T cells were transfected with the indicated Flag-tagged Mdm2 constructs and HA-SUMO-1. WCE were immunoprecipitated with antibodies indicated in the figure. The two bottom panels verify expression of the respective exogenously expressed constructs. The top panel shows conjugation of wt Mdm2 as well as K1 (K446R), K2 (K453R), and K3 (K465,466,468,469,472R) mutants to SUMO-1.
(B) Purified GST-tagged Mdm2 forms were sumoylated in vitro using in vitro translated $^{35}$S-labeled SUMO-1. The MW of the sumoylated Mdm2 is 120 kDa, due to the GST moiety. After the sumoylation reaction, GST-bound proteins were separated on SDS-PAGE and visualized by Coomassie staining (bottom panel). Upper panel shows an autoradiograph of sumoylated Mdm2 forms. Efficient sumoylation of Mdm2 was seen with the wt, K2, or K3 mutants but not with the K1 mutant form or in the absence of SUMO-1. Reduced sumoylation was seen in the absence of Ubc9 because of the presence of minute amounts of the Ubc9 in the wheat germ extract used to perform in vitro translation.

We next examined whether SUMO-1 modification alters Mdm2 self-ubiquitination by performing the sumoylation reaction prior to in vitro ubiquitination. SUMO-1 modification of Mdm2 was performed with purified bacterially expressed components, including SUMO-1, Aos1/Uba2, and Ubc9. As shown in Figure 3A, sumoylation of the wt Mdm2 efficiently decreased the degree of

Mdm2 self-ubiquitination (Figure 3A, lane 1 versus lane 3). The efficiency of Mdm2 sumoylation under these conditions was verified by slower migration of SUMO-1-Mdm2 (Figure 3A, lower panel). Taken together, these data demonstrate that Lys-446 serves as the primary ubiquitination site on Mdm2 and that Mdm2 sumoylation attenuates its self-ubiquitination in vitro.

### Lysine 446 Is Required for Mdm2 Ubiquitination and Degradation In Vivo

We next examined the role of Lys-446 in Mdm2 ubiquitination in vivo. Expression of Flag-tagged Mdm2 and HA-tagged ubiquitin in 293T cells followed by Flag immunoprecipitation and anti-HA antibody analysis enabled the detection of ubiquitinated wt Mdm2 (Figure 3B). In contrast, K446R mutant was not ubiquitinated. These observations establish that Lys-446 is required for Mdm2 ubiquitination in vivo.

To determine whether self-ubiquitination of Mdm2 at Lys-446 is required for Mdm2 degradation in vivo, we measured Mdm2 half-life. $^{35}$S-pulse-chase labeling experiments revealed that the 90 kDa form of Mdm2 is more stable compared with the 75 kDa form of Mdm2 (Figure 3C). Whereas the half-life of the K1 form is around 2 hr, the p90 sumoylated form of Mdm2 exhibits a half-life of less than 1 hr as compared with a half-life of less than 30 min for the non-SUMO-1-modified p75 form of Mdm2. Treatment of the cells with proteasome inhibitor lactacystin prolonged the half-life of both p75 and p90, whereas the half-life of the K1 remained unchanged (Figure 3C). These observations further substantiate that conjugation of SUMO-1 to Mdm2 stabilizes Mdm2 by preventing its self-ubiquitination. It should be noted that since the Mdm2 of ~90 kDa is sumoylated (Figures 1–2) and its direct ubiquitination may be impaired (Figure 3A), it is likely that removal of SUMO-1 by sentrin-specific protease1-like enzymes occurs prior to Mdm2 ubiquitination. In this case, the difference in the kinetics of p90 form of Mdm2 and K1 degradation is probably underestimated. Together with the data presented in Figure 3B, these results suggest that Lys-446 is required for Mdm2 ubiquitination and degradation in vivo.

### Mdm2 Sumoylation Does Not Affect Its Association with p53

We next determined whether SUMO-1 conjugation to Mdm2 affects Mdm2 binding to p53. In its sumoylated form, Mdm2 is as efficient in its association with p53 as the mutant form, as revealed by immunoprecipitation of Mdm2 with antibodies to p53, or vice versa (Figure 4A). The association between p53 and Mdm2 could be identified using antibodies to either the amino or the carboxyl terminus of Mdm2, thus excluding the presence of a spliced Mdm2 form (Perry et al., 2000) in this complex. Each of the Mdm2 forms tested was capable of associating with p53 in vitro as evident from coimmunoprecipitation of in vitro translated proteins (Figure 4B). Forced expression of p53 and wt or the K1 forms of Mdm2 in p53/mdm2 double null cells also enabled the detection of efficient association between p53 and the wt (sumoylated in cells) or K1 (non-SUMO-1-modified) forms of Mdm2 (Figure 4C). These results suggest that SUMO-1 modification does not affect Mdm2 association with p53.

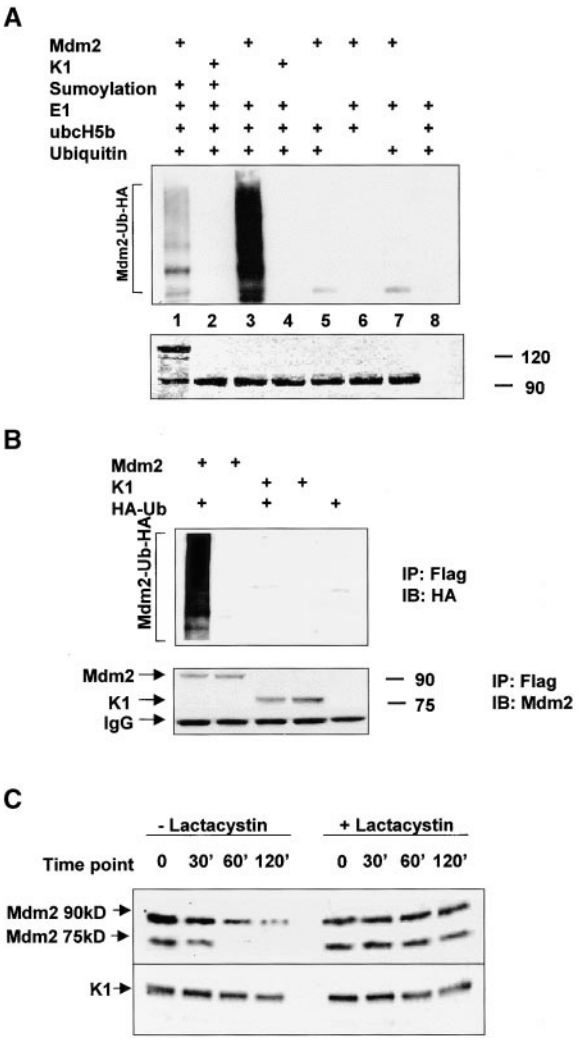


Figure 3. Sumoylation at Lys-446 of Mdm2 Abolishes Its Self-Ubiquitination and Increases Its Stability

(A) Wt or K1 mutant forms of Mdm2 were subjected to the sumoylation reaction using purified SUMO-1, Aos1/Uba2, and Ubc9 as detailed in the Experimental Procedures. SUMO-1- (or mock)-modified forms of Mdm2 were then subjected to the self-ubiquitination assay using $^{32}$P-ubiquitin in the presence of purified E1 and E2. Following the ubiquitination reaction, glutathione bead-coupled proteins were washed, and proteins were separated on 8% SDS-PAGE. The lower panel depicts Mdm2 protein visualized by Coomassie staining. The upper panel shows an autoradiograph of $^{32}$P-ubiquitin conjugated to the respective Mdm2 forms.

(B) 293T cells were transfected with the wt or K1 forms of Mdm2 and HA-Ub. WCE was subjected to immunoprecipitation using monoclonal antibodies to the Flag-tag. The lower panel shows expression level of the transfected Mdm2 forms. The upper panel depicts poly-HA-Ubiquitin chains conjugated to the respective Mdm2 forms.

(C) p53/Mdm2 double null cells were transfected with the wt or K1 forms of Mdm2 and 24 hr later were subjected to pulse chase labeling with $^{35}$S-methionine in the absence or presence of lactacystin (25 μM) as indicated in the figure. Proteins prepared at the indicated time points were immunoprecipitated using monoclonal antibodies to Mdm2 followed by autoradiography. The upper part of the figure depicts analysis of the wt Mdm2-transfected cells whereas the lower part shows analysis of the K1-expressing cells.

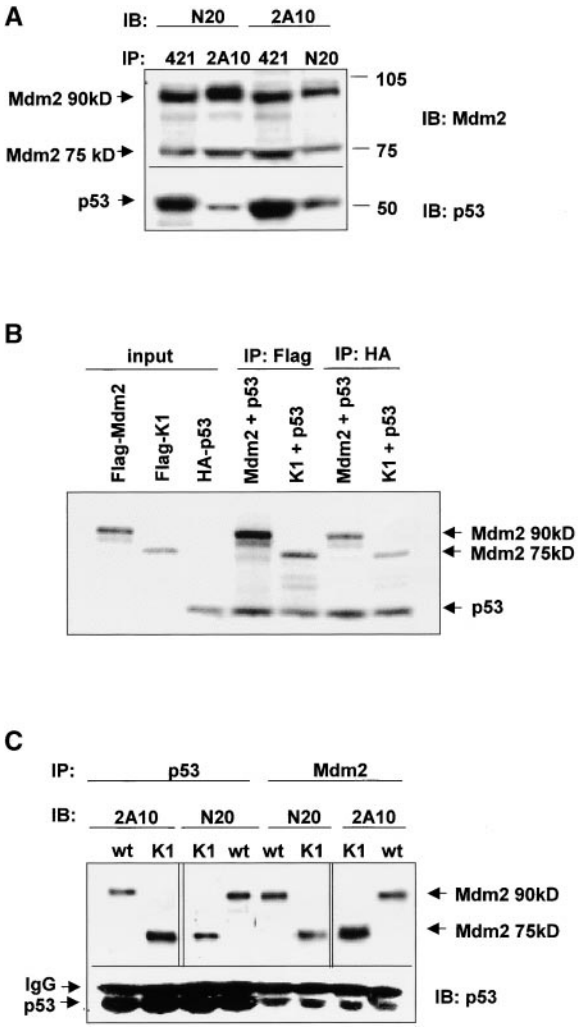


Figure 4. Mdm2 Sumoylation Does Not Affect Its Association with p53

(A) The association of Mdm2 with p53 is not affected by its sumoylation. Immunoprecipation of p53 (pAb421) or of Mdm2 (2A10 to C- and N20 to N-terminal domains) followed by immunoblot analysis using either of the Mdm2 antibodies (upper panel) or p53 antibodies (lower panel) identified the association of p53 with Mdm2 in its SUMO-1-modified form and in its nonsumoylated form.

(B) Mdm2 in its wt or K1 forms associates with p53 in vitro. In vitro translated, $^{35}$S-labeled, Flag-tagged Mdm2 wt or K1 forms were incubated with HA-tagged p53 followed by immunoprecipitation with antibodies to either p53 (HA) or Mdm2 (Flag). Immunopurified material was separated on SDS-PAGE and subjected to autoradiography. Immunoprecipitations identified both the target and associated protein, respectively, as indicated by arrows on the right panel.

(C) Wt or K1 forms of Mdm2 associate with p53 in vivo. Forced expression of Flag-tagged Mdm2 wt or K1 and HA-tagged p53 in Mdm2/p53 double null cells was followed by immunoprecipitation and immunoblot analysis with the antibodies indicated in the figure.

### Mdm2 K446R, which Is Not Capable of Self-Ubiquitination, Is More Efficient than Wt Mdm2 in Promoting p53 Degradation

Mdm2 elicits the ubiquitin ligase activities toward itself as well as toward p53. Given the observations that Mdm2 sumoylation attenuates its self-ubiquitination and prolongs its half-life but does not affect its association with p53, we have examined the significance of Mdm2 self-ubiquitination in the Mdm2-mediated degradation of p53. To this end, we compared the effects of the wt Mdm2 and the K1 mutant on p53 accumulation in a cotransfection assay (Haupt et al., 1997; Kubbutat et al., 1997). p53/Mdm2 double null cells (Jones et al., 1995) were used to exclude the influence of endogenous Mdm2. Forced expression of the wt or the K1 forms of Mdm2 in these cells led to a dose-dependent decrease in the levels of p53 protein. Nevertheless, when compared with the wt form of Mdm2, five times lesser amounts of K1 expression construct were required to achieve the same decrease in the level of p53 (0.5 versus 2.5 μg; Figure 5A, upper panel). This result indicates that the Mdm2 mutant defective in self-ubiquitination is more efficient in promoting p53 degradation in vivo than the wt Mdm2. Since, under the conditions of forced Mdm2 expression used in these experiments, the levels of K1 and wt forms of Mdm2 were similar (Figure 5A, lower panel), we cannot explain the enhanced activity of K1 by merely the accumulation of a more stable K1 protein. Therefore, these data also suggest that Mdm2 self-ubiquitination is likely to be detrimental to Mdm2's ability to ubiquitinate p53.

### Sumoylation Increases the Ability of Mdm2 to Ubiquitinate p53

To directly assess the effect of Mdm2 ubiquitination on its ability to target the degradation of p53, we performed the Mdm2 self-ubiquitination reaction prior to adding Mdm2 to p53 ubiquitination assay. In vitro ubiquitination reactions were carried out using the purified E1, E2 (UbcH5b), Mdm2 (as the source for E3 ligase), and affinity-purified HA-tagged p53 expressed in Sf9 insect cells. In this two-step reaction, we enabled efficient self-ubiquitination of Mdm2 before addition of p53 as a substrate to assay Mdm2-targeted ubiquitination. Analysis of p53 ubiquitination (performed with antibodies to HA-tagged p53, which enabled distinguishing p53 from Mdm2 ubiquitination) revealed that in its ubiquitinated form Mdm2 is less efficient in mediating the ubiquitination of p53 (Figure 5B).

We have next assessed the effect of Mdm2 sumoylation on its ability to target the ubiquitination of p53. Whereas Mdm2 mediated noticeable p53 ubiquitination (which was dependent on the presence of E1, E2, ubiquitin, and ATP; Figure 5B; data not shown), there was a remarkable increase in the degree of p53 ubiquitination by SUMO-1-modified Mdm2 (Figure 5C). An increased ubiquitin ligase activity of the K1 mutant correlates with its elevated ability to degrade p53 in vivo (Figure 5A). Sumoylated Mdm2 was as efficient in p53 ubiquitination as the K1 mutant of Mdm2, which does not exhibit self-ubiquitination. These data suggest that whereas ubiquitination of Mdm2 impairs its ubiquitin ligase activity, Mdm2 sumoylation at Lys-446 preserves Mdm2 ligase activity for p53 ubiquitination by blocking the self-ubiquitination of Mdm2 E3 ubiquitin ligase.

### Mdm2 K446R Is More Efficient than Wt Mdm2 in Inhibition of p53-Induced Apoptosis

To examine the biological consequences of accelerated p53 degradation by mutant Mdm2, deficient in self-ubiquitination, we compared the abilities of the K1 and

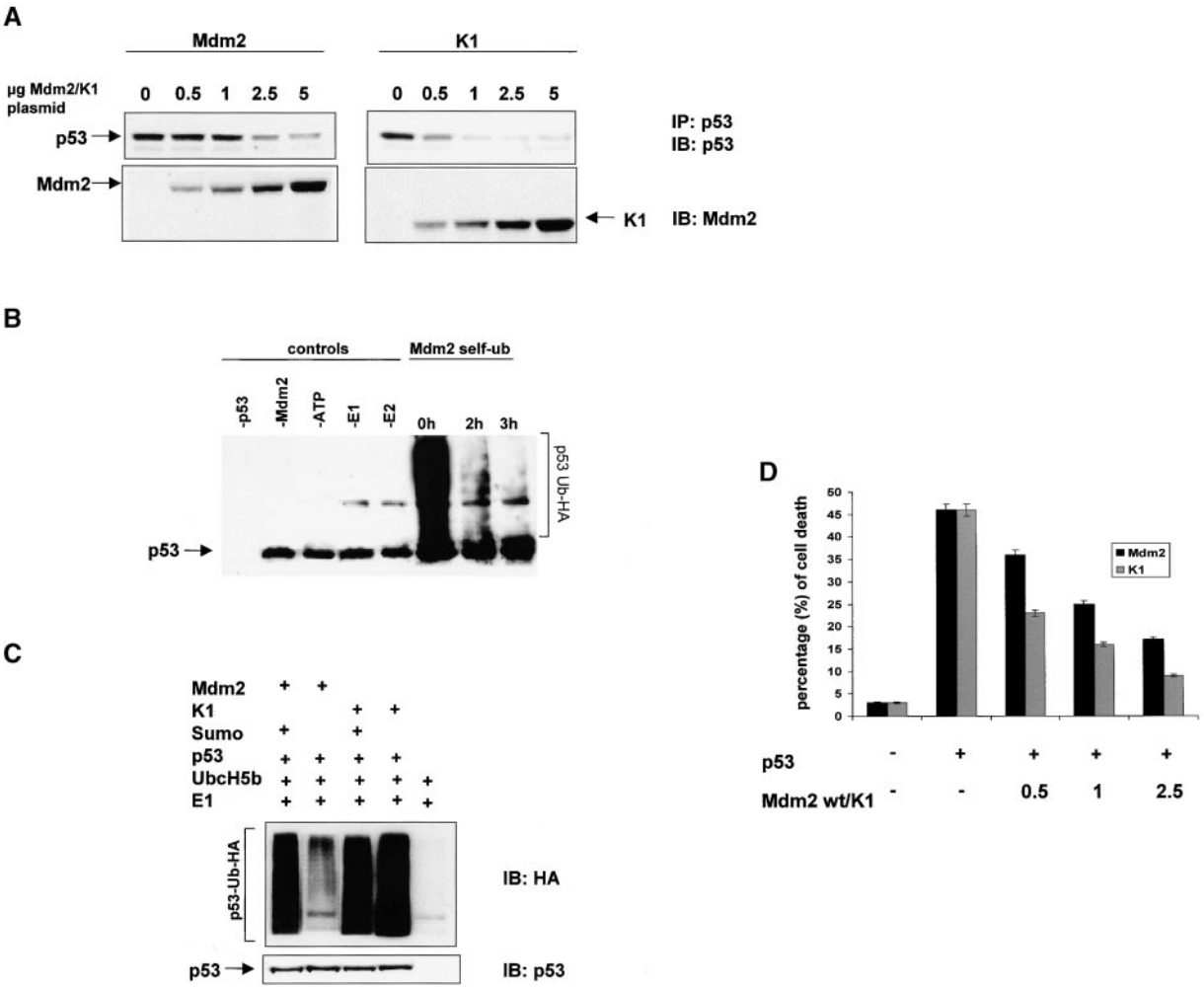


Figure 5. Mdm2 Sumoylation or Mutation on Lys-446 Increases p53 Ubiquitination and Degradation and Decreases p53 Ability to Mediate Apoptosis

(A) Mdm2/p53 double null cells were transfected with p53 and increasing amounts of the wt or the K1 forms of Mdm2 as indicated. WCE was immunoprecipitated using monoclonal p53 antibodies. The lower panel shows expression levels of wt or K1 forms of Mdm2 detected by monoclonal Mdm2 antibodies. The upper part of the figure shows the relative decrease in p53 expression levels upon Mdm2 (wt versus K1) expression.

(B) Ubiquitinated Mdm2 is less efficient in targeting the ubiquitination of p53. Bacterially expressed Mdm2 (3 μg) was subjected to in vitro ubiquitination (self-ubiquitination) in the presence of E1 (45 ng), E2 UbcH5b (1.5 μg), and ubiquitin (3 μg; SIGMA) and 2 mM ATP in ubiquitination buffer for the 0, 2, or 3 hr (to ensure efficient self-ubiquitination) as indicated in the figure. To monitor the degree of Mdm2's ability to target the ubiquitination of p53, equal amounts of the self-ubiquitinated Mdm2 were extensively washed with TBS/0.5% Triton X-100 before being incubated with bacculovirus-expressed and purified form of HA-tagged p53 for 2 hr in the presence of E1, E2, and ubiquitin. The degree of p53 ubiquitination was monitored via immunoblot analysis using antibodies to HA.

(C) Wt or K1 mutant forms of GST-Mdm2 (1 μg) were sumoylated (in the presence of purified E1 [Aos1/Uba2; 15 ng], E2 [Ubc9; 0.5 μg] and SUMO-1 [1 μg]) or mock treated before incubation for 1 hr with WCE (1 mg) prepared from 293T cells that overexpress exogenous forms of HA-p53. GST-Mdm2-p53-bound beads were washed with TBS/0.5% Triton X-100 to minimize nonspecific binding before initiating the in vitro ubiquitination reaction in the presence of E1 (15 ng), E2 UbcH5b (0.5 μg), and ubiquitin (1 μg; SIGMA) and 2 mM ATP in ubiquitination buffer. Proteins were separated on 10% SDS-PAGE. The ubiquitinated HA-p53 was detected by monoclonal HA-antibodies. Similar observations were made using HA-tagged p53 that was purified from bacculovirus-expressing Sf9 cells (data not shown).

(D) DNA fragmentation analysis of Mdm2/p53 double null cells cotransfected with wild-type p53, increasing concentrations of wt, or K1 forms of Mdm2, and GFP plasmids. Percent of apoptotic GFP-positive cells is indicated in the figure.

wt forms of Mdm2 to inhibit p53-mediated apoptosis. Coexpression of wt or K1 forms of Mdm2 in Mdm2/p53 double null cells resulted in a dose-dependent decrease in the apoptosis induced by forced expression of p53. When compared with wt Mdm2, the K1 mutant, which is protected from self-ubiquitination, elicited a greater decrease in degree of cell death (Figure 5D). This finding suggests that abolished self-ubiquitination renders Mdm2 more efficient in inhibiting p53-mediated programmed cell death, possibly because of the accelerated degradation of p53.

### Radiation Decreases the Degree of Mdm2 Sumoylation

Stabilization of p53 in response to stress and DNA damage is imperative for its ability to coordinate the cellular

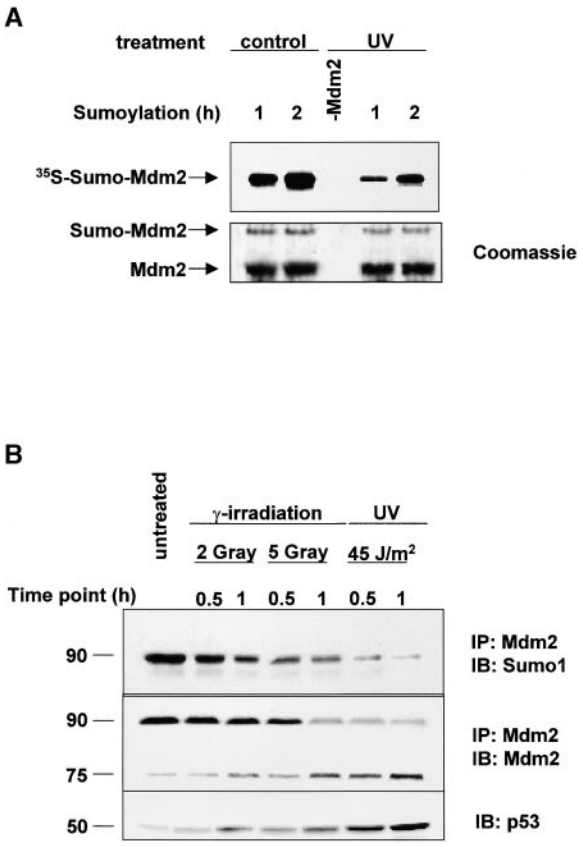


Figure 6. Mdm2 Sumoylation Is Reduced after Cell Exposure to Radiation

(A) Proteins prepared from control (-UV) or 2 hr after UV-irradiation were incubated with bacterially expressed Mdm2 and in vitro translated $^{35}$S-labeled SUMO-1 under conditions required to enable Mdm2 sumoylation (for 1 or 2 hr as indicated in the figure). The degree of SUMO-1 conjugation was monitored via autoradiography of the SDS-PAGE (upper panel), whereas the amount of Mdm2 in its respective forms was visualized via Coomassie blue staining (lower panel). Compare changes between control and UV treatment at the same time point, respectively.

(B) Normal human fibroblasts were treated with sham, UV, or X-rays at indicated doses in the presence of proteasome inhibitor (MG132, 40 μM, for 3 hr before protein preparation). Proteins, prepared at the indicated time points after exposure, were immunoprecipitated with antibodies to Mdm2 (IF2, which were used in this experiment, rather than 2A10 antibodies used in former experiments, since the latter do not recognize Mdm2 after exposure to radiation) and subjected to immunoblot analysis using antibodies to SUMO-1 (upper part of the figure). Middle part of the figure depicts analysis of the same membrane following its incubation with antibodies to Mdm2. Lower part of the figure shows immunoblot of p53 in extracts (100 μg) prepared from cells subjected to the same treatments yet in the absence of the proteasome inhibitor.

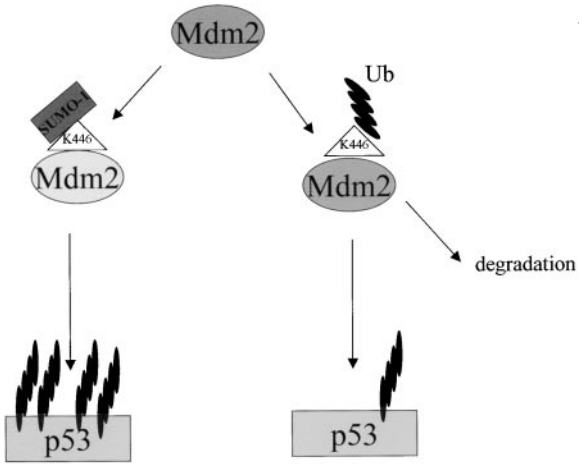


Figure 7. Model for the Regulation of Mdm2 Ubiquitin-Ligase Activity by SUMO-1

Mdm2 is subjected to either ubiquitination or sumoylation. When self-ubiquitinated, the ubiquitin ligase activity of Mdm2 for p53 is impaired. Upon SUMO-1 conjugation, Mdm2 is protected from ubiquitination and elicits increased ubiquitin ligase activity, as reflected in increased ubiquitination and degradation of p53.

stress response. Under these circumstances, increased p53 stability has been attributed, in part, to its dissociation from Mdm2 (Shieh et al., 1997; Fuchs et al., 1998). The current finding of Mdm2 sumoylation, and its effect on Mdm2 E3 ligase activity toward p53, points to an additional layer of regulation, which is independent of Mdm2 dissociation from p53. Given the effect of sumoylated Mdm2 on p53 ubiquitination, we determined whether DNA damage, which is among the best characterized stimuli that increase p53 stability, alters the relative amount of SUMO-1-modified Mdm2. Proteins prepared from UV-treated cells were tested for their ability to mediate Mdm2 sumoylation in vitro. When compared with proteins prepared from nontreated cells, the proteins derived from UV-treated cells were less efficient in eliciting Mdm2 sumoylation (Figure 6A). These observations suggest that degree of Mdm2 sumoylation is reduced upon UV-irradiation. Mdm2 that has been subjected to sumoylation in the presence of proteins from UV-treated cells also elicited a less efficient ubiquitination of p53 than proteins prepared from sham-treated cells (data not shown).

To further assess the changes to Mdm2 sumoylation in response to radiation, we have monitored the expression levels of the SUMO-1-modified Mdm2, the nonsumoylated form of Mdm2, and p53 in proteins prepared from UV- or X-ray-treated cells. A clear time- and dose-dependent decrease in the amount of SUMO-1-modified Mdm2 after exposure to either UV or X-rays was seen both in immunoprecipitation of Mdm2 followed by SUMO-1 Western (Figure 6B, upper panel) and in straight immunoblot analysis of 90 kDa Mdm2 (Figure 6B, middle panel). Immunoblot analysis of Mdm2 expression using proteins prepared after UV or X-ray treatment in the presence of proteasome inhibitors (to preserve the nonsumoylated form of Mdm2) revealed a shift from the sumoylated to the nonsumoylated forms of Mdm2, in a time- and dose-dependent fashion (Figure 6B, middle panel). These results suggest that DNA damage, in the form of radiation, effectively diminishes the degree of Mdm2 sumoylation in a dose- and time-dependent manner. The decrease of Mdm2 sumoylation coincided with an increased level of p53 expression (Figure 6B, lower panel), suggesting that the regulation of SUMO-1 modification of Mdm2 may play a role in p53 stabilization in response to DNA damage. The temporal decrease in the amount of SUMO-1-conjugated Mdm2 points to one of the mechanisms by which p53 is no longer targeted by Mdm2 for ubiquitination in response to DNA damage.

## Discussion

A common characteristic of RING finger proteins that exhibit ubiquitin ligase activity is that they are capable of self-ubiquitination (Lorick et al., 1999; Fang et al., 2000; Honda and Yasuda, 2000). While it is conceivable that self-ubiquitination is required to limit the duration and magnitude of ubiquitin ligase output, the mechanism distinguishing self-ubiquitination from the ubiquitination of the target substrate has long been sought. Such a mechanism would have been expected to provide a window of opportunity for substrate ubiquitination before self-destruction takes place.

Here, we demonstrate that self-ubiquitination of Mdm2 at Lys-446 within its RING finger domain impairs Mdm2 ubiquitin ligase activity and ability to ubiquitinate p53. We also show that conjugation of the SUMO-1 ubiquitin-like protein to the same lysine residue abrogates Mdm2 self-ubiquitination and therefore preserves Mdm2 ubiquitin ligase activity, resulting in concomitantly increased efficiency of ubiquitination of its major substrate, p53 (Figure 7). Although the abilities of sumoylated Mdm2 and self-ubiquitination-deficient K1 mutant to ubiquitinate p53 were similar, we cannot rule out the possibility that in addition to preventing self-ubiquitination, sumoylation elevates Mdm2 activity by another mechanism. Our data provide additional evidence for the important role of ubiquitin-like proteins in the modulation of E3 ubiquitin ligase activities. Conjugation of Cullins with the ubiquitin-like protein Nedd8/RUB1 is implicated in the biological activities of other RING finger-based E3 ubiquitin ligases such as SCF-ROC1 and VCB-Cul2-ROC1 (Lammer et al., 1998; Kamura et al., 1999; Hori et al., 1999; Liakopoulos et al., 1999). Like Mdm2 sumoylation, Nedd8/RUB1 conjugation is also capable of activating the ability of SCF/$\beta$T$\gamma$CP to ubiquitinate I$\kappa$B$\alpha$ (Read et al., 2000) this modification has yet to be identified.

Sumoylation of PML or Sp100 proteins was reported to result in nuclear localization within PML bodies (Duprez et al., 1999; Strensdorf et al., 1999). Subcellular localization of Mdm2 is unlikely to be affected by its sumoylation since the localization of the K1 mutant that cannot undergo SUMO-1 modification did not differ from the localization of the wt protein (data not shown). This finding suggests that protein sumoylation may result in multiple biological outcomes, varying with the nature of the sumoylated protein.

In the case of Mdm2, SUMO-1 modification competes with Mdm2 ubiquitination for the major conjugation site, Lys-446. It was previously shown that similar competition between sumoylation and ubiquitination occurs at the Lys-21 of I$\kappa$B$\alpha$ (Desterro et al., 1998). Although resembling the case of I$\kappa$B$\alpha$ in the role of the active interplay between sumoylation and ubiquitination dictating the degree of Mdm2 stability, SUMO-1 modification of Mdm2 has major consequences for its activity as ubiquitin ligase. Moreover, unlike the sumoylation of I$\kappa$B$\alpha$, which was shown to occur in a small percentage of I$\kappa$B$\alpha$ molecules (Desterro et al., 1998), SUMO-1 modification of Mdm2 was found in almost 100% of exogenously expressed proteins and is largely noted among the endogenous Mdm2 species (as represented by the 90 kDa MW band). Indeed, Mdm2 has often been referred to as an $\sim$90 kDa protein (Chen et al., 1993, 1996). Our experiments suggest that the nonsumoylated form of Mdm2, which was bound to p53 and was recognized by amino-terminal Mdm2 antibodies, is not a spliced form of Mdm2, which lacks N-terminal sequences (Perry et al., 2000). Given that sumoylation protects the Mdm2 protein from degradation, SUMO-1-modified Mdm2 is expected to be the more abundant Mdm2 form. That a large portion of Mdm2 molecules are sumoylated further emphasizes the biological significance of SUMO-1 modification for the Mdm2 protein.

Our findings demonstrate that sumoylation resulted in the inhibition of Mdm2 self-ubiquitination, thus providing the mechanism that enabled us to distinguish between substrate ubiquitination and self-ubiquitination. Accordingly, duration of protein sumoylation is expected to reflect the time frame during which the ubiquitination of Mdm2 targets (such as p53) occurs. A temporal decrease in Mdm2 sumoylation occurs in response to DNA damage and is inversely correlated with the elevated levels of p53. Attenuated Mdm2 sumoylation is likely to explain why Mdm2 is no longer capable of mediating the efficient ubiquitination of p53 in response to DNA damage, in addition to decreased p53-Mdm2 binding (Shieh et al., 1997; Fuchs et al., 1998).

Maintenance of a sumoylated substrate is expected to be regulated by the balance between Mdm2 sumoylation and removal of SUMO-1 by the desumoylating enzymes, including the sentrin-specific protease 1 (SENP1), which is distantly related to the yeast Smt3-specific protease ULP1 (Li and Hochstrasser, 1999; Gong et al., 2000). Regulation of Mdm2 sumoylation may also be affected by Mdm2 phosphorylation as well as by some of its associated proteins, including $p14^{ARF}$, p300, pRb, and E2F1.

The consequences of Mdm2 sumoylation, as shown in this study, are reflected in increased ubiquitination and degradation of p53 and the diminished p53 ability to elicit programmed cell death, which results in attenuated activities of this tumor suppressor protein. Accordingly, sumoylation is likely to play a key role in Mdm2's ability to elicit its oncogenic activities.

Sumoylation of the Mdm2 target substrate p53 was shown not to affect p53 stability and was found on only a small portion of p53 molecules (Gostissa et al., 1999; Rodriguez et al., 1999), suggesting that SUMO-1 modification of Mdm2 may have greater implications for the biological activities of p53.

All in all, our findings that Mdm2 sumoylation increases its ubiquitin ligase activities while protecting Mdm2 from self-ubiquitination provide an important example of the contribution of SUMO-1 modification to the maintenance of intrinsic E3 ubiquitin ligase activity.

### Experimental Procedures

#### Expression Vectors

Human wt Mdm2 cDNA was amplified by PCR (HiFi Taq polymerase, GIBCO) and unidirectionally cloned, with the addition of two N-terminal Flag-tags, between the EcoRI and BamHI sites of pcDNA3. Bacterial expression vector of GST-Mdm2 (in pGEX-4T-1) was kindly provided by Dr. A. Weissman. Wt Mdm2 in pcDNA3 or pGEX-4T-1 was used as a backbone for generating the site-directed mutagenesis (Quick Change, Stratagene) to substitute lysines 446 (K1), 453

(K2) or 465, 466, 468, 469, 472 (K3) for arginines. The integrity of the resulting constructs was verified by sequencing. HA-tagged p53 expression vector was previously described (Fuchs et al., 1998). pcDNA3-HA-SUMO-1 expression vector was kindly provided by Dr. R. Hay (Rodriguez et al., 1999). PCMV-HA-Ub expression construct was a gift from Dr. D. Bohmann (Treier et al., 1994).

### Cells

293T, adenovirus-transformed human embryo kidney cells (a kind gift of Dr. E. Spanopoulou), p53/Mdm2 double null cells (kindly provided by Dr S. Jones), and normal human fibroblasts (a kind gift of Dr. H. Tahara) were maintained in DMEM (GIBCO), supplemented with 10% heat-inactivated fetal bovine serum and antibiotics in 5% $CO_2$. Transfections were performed by the calcium phosphate precipitate technique for 293T cells and by lipofection (Lipofectamin-Plus, GIBCO) for p53/Mdm2 null cells. The medium was changed 6 hr after transfection, and cells were harvested 24 hr later.

### Antibodies

Monoclonal antibodies against Flag- and HA-epitopes were purchased. (M2, SIGMA, and HA11, BabCo). Antibodies (monoclonal) against SUMO-1 were purchased (Zymed). Monoclonal antibodies 2A10 (a gift from A. Levine), N20 (Santa Cruz), and IF2 (Oncogene) were used for Mdm2 analysis. p53 detection was carried out using monoclonal pAb421 antibodies (Oncogene) and polyclonal FL393 antibodies (SantaCruz).

### In Vitro Conjugation of SUMO-1

Glutathione beads (SIGMA) were used to purify bacterially expressed forms of GST-Mdm2 forms (wt, K1, K2, K3) as previously described (Lorick et al., 1999). Bacterially expressed purified Mdm2 (1–2 μg) proteins were incubated with either purified form of bacterially expressed SUMO-1 or with in vitro translated $^{35}$S-labeled SUMO-1 (TNT, Promega), as indicated in the Results. To initiate SUMO-1 conjugation to Mdm2, purified Aos1/Uba2 (15 ng) and Ubc9 (0.5 μg) were added to the reaction mix for 30 min at 37°C in conjugation buffer (20 mM Hepes [pH 7.4], 5 mM $MgCl_2$, 2 mM ATP, 10 mM creatine phosphate, and 1 unit creatine phosphokinase). Bead-bound SUMO-1-modified Mdm2 was either taken for subsequent reaction (see p53 ubiquitination) or washed (3× TBS/0.5% Triton-X-100) before denaturation for 5 min at 95°C in 3× Sample Buffer. Proteins were separated on 8% SDS-PAGE. The gel was stained with Coomassie blue, dried, and exposed to X-ray film (Xomat, Kodak).

### Mdm2 Self-Ubiquitination Assay

Purified GST-Mdm2 wt or mutant forms were sumoylated (or mock treated with the respective buffers as control) by addition of the purified form of bacterially expressed SUMO-1 together with Aos1/Uba2 and Ubc9 (see in vitro sumoylation reaction). Upon its SUMO-1 modification, Mdm2 bound to glutathione beads was washed and incubated for 1 hr at 37°C in ubiquitination buffer (50 mM Tris-HCl [pH 8.0], 5 mM $MgCl_2$, 0.5 mM DTT, 2 mM NaF, and 3 mM okadaic acid) supplemented with $^{32}$P-labeled his-tagged ubiquitin (Tan et al., 1999), purified E1 (15 ng), UbcH5b (0.5 μg), and ATP (2 mM). Glutathione-bound GST-Mdm2 beads were extensively washed (3× with TBS/0.5% Triton-X-100). To analyze Mdm2 self-ubiquitination, the proteins remaining on the washed beads were eluted in Laemmli buffer, separated on 8% SDS-PAGE, and analyzed by autoradiography and Coomassie Blue staining.

### In Vitro Ubiquitination of p53

Purified GST-Mdm2 forms that were sumoylated or mock treated (with all reagents except SUMO-1) were incubated for 1 hr on ice with HA-p53 that was either affinity purified from Sf9 cells infected by bacculovirus expressing HA-p53 (a gift of Dr. P. Tegtmeyer) or with protein extracts prepared from 293T cells that overexpress HA-p53. Bead-bound SUMO-1-modified (or not) Mdm2-p53 complex was washed (3× with TBS/0.5% Triton-X-100) before incubation with purified E1 (15 ng), Ubc9 (0.5 μg), ATP (2 mM), and ubiquitin (1 μg, SIGMA) for 30 min at 37°C. Glutathione-bound GST-Mdm2-p53 beads were washed (3× with TBS/0.5% Triton-X-100), eluted by Laemmli sample buffer, separated by 10% SDS-PAGE, and analyzed by immunoblotting using monoclonal HA-antibody.

### In Vivo Half-Life Measurements

p53/Mdm2 double null cells were transfected with wt or K1 forms of Mdm2 expression vectors (4 μg). $^{35}$S-methionine (1 mCi) was added to the cell cultures 24 hr after transfection for 5 hr (pulse) followed by chase (2 mM cold methionine) for the indicated time points. Protein extracts (1 mg) were used for immunopurification of Mdm2 using mixture of monoclonal antibodies (2A-10/IF2). Immunoprecipation Mdm2 was washed, separated on 10% SDS-PAGE, and analyzed by autoradiography.

### In Vivo Sumoylation/Ubiquitination

Cells were transfected with Mdm2 constructs and cDNAs encoding either HA-tagged SUMO-1 or HA-tagged ubiquitin to analyze sumoylation or ubiquitination, respectively. Harvested cell pellets were lysed by incubation with two volumes of 2% SDS in TBS (10 mM Tris-HCl [pH 8.0] and 150 mM NaCl) at 95°C for 10 min. Eight volumes of 1% Triton-X-100 in TBS were added, and lysates were sonicated for 2 min. The solution was incubated for 30 min at 4°C with Protein A/G beads (GIBCO) and clarified by 30 min centrifugation (14,000 rpm) at 4°C. The protein concentration was determined by Bradford assay. For immunoprecipation, 500 μg of protein was incubated with respective antibodies at 4°C overnight before Protein A/G beads (25 μl) were added for 2 hr. The beads were washed with 0.5 M LiCl in TBS followed by two additional washes with TBS. Proteins were loaded onto 10% SDS-PAGE, followed by immunoblot analysis with the indicated antibodies and ECL detection (Amersham).

### Apoptosis Assay

Mdm2/p53 double null cells were transfected with Mdm2 constructs (as indicated) and marker plasmid encoding Green Fluorescent Protein, pEGFP (1 μg). Twenty-four hours after transfection, cells were collected, fixed in 1% paraformaldehyde, treated with 70% ethanol, washed with PBS, treated with DNase-free RNase A (1 mg/ml), and stained with propidium iodide ([PI] 40 μg/ml). DNA fragmentation analysis of GFP-positive cells (50,000 per measurement in triplicates) was carried out on a flow cytometer (Becton Dickinson). The percentage of cells to the left of the diploid $G_{0/1}$ peak, characteristic of hypodiploid cells, was calculated as the percentage of apoptotic cells.

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