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Mdm2-Mediated NEDD8 Conjugation of p53 Inhibits Its Transcriptional Activity

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Summary

The only reported role for the conjugation of the NEDD8 ubiquitin-like molecule is control of the activity of SCF ubiquitin ligase complexes. Here, we show that the Mdm2 RING finger E3 ubiquitin ligase can also promote NEDD8 modification of the p53 tumor suppressor protein. Mdm2 is itself modified with NEDD8 with very similar characteristics to the autoubiquitination activity of Mdm2. By using a cell line (TS-41) with a temperature-sensitive mutation in the NEDD8 conjugation pathway and a p53 mutant that cannot be NEDDylated (3NKR), we demonstrate that Mdm2-dependent NEDD8 modification of p53 inhibits its transcriptional activity. These findings expand the role for Mdm2 as an E3 ligase, providing evidence that Mdm2 is a common component of the ubiquitin and NEDD8 conjugation pathway and indicating the diverse mechanisms by which E3 ligases can control the function of substrate proteins.

Introduction

Ubiquitin is the founder member of the family of ubiquitin-like proteins. These are small proteins that are covalently ligated to other proteins via an isopeptide linkage between the C-terminal carboxyl group of ubiquitin-like proteins and the ϵ-amino group of a lysine residue in the modified protein (Ciechanover, 1998). In addition to ubiquitin, the family of ubiquitin-like proteins includes ubiquitin crossreactive protein (UCRP or ISG15), SUMO-1 (also known as Smt3c, GMP1, or sentrin/Ubl1/Pic1), NEDD8 (also known as Rub1), and Apg12. While ubiquitination can target proteins for proteasomal degradation, conjugation of ubiquitin-like molecules has novel regulatory functions (Weissman, 2001).

Among the ubiquitin-like protein family, NEDD8 is most homologous to ubiquitin. NEDD8 conjugation is mechanistically similar to ubiquitination, in that NEDD8 is activated and transferred to substrates by E1 and E2 enzymes. Unlike the ubiquitin E1 enzyme, which is a single polypeptide, the NEDD8 E1 enzyme is a heterodimer composed of the amyloid precursor protein binding protein (APP-BP1) and Uba3 protein. The E2 conjugating enzyme for NEDD8 is the Ubc12 E2-like protein (Osaka et al., 1998; Gong and Yeh, 1999). Genetic studies in plant, animal, and fungal systems have highlighted the importance of the NEDD8 conjugation pathway in cell proliferation, viability, and development. In *Arabidopsis*, mutations in the *AXR1* gene (the homolog of *APP-BP1*) result in defects in a variety of auxin-regulated processes (Cernac et al., 1997; Pozo et al., 1998). Auxin is one of the most extensively studied plant hormones, which regulates cell division, cell elongation, and cell differentiation. In *Saccharomyces cerevisiae* and in fission yeast, the NEDD8 modifying pathway is essential for cell growth and viability, respectively (Lammer et al., 1998; Osaka at al., 2000). In addition, a temperature-sensitive mutation in the *SMC* gene, the hamster homolog of human APP-BP1, is responsible for the dramatic cell cycle defects observed in TS-41 hamster cells. At the restrictive temperature, these cells undergo multiple rounds of DNA replication without intervening mitoses (Handeli and Weintraub, 1992). Further evidence for the importance of the NEDD8 pathway for proliferation in mammals came from studies in mice (Tateishi et al., 2001). Deletion of the *Uba3* gene, encoding for the catalytic component of the NEDD8-activating heterodimer enzyme, caused embryonic death. At the molecular level, the only well-characterized substrate so far for NEDD8 conjugation (NEDDylation) is members of the cullin family of proteins (Lammer et al., 1998; Liakopoulos et al., 1998; Osaka et al., 1998), although recent studies suggest that the cytoskeleton could be another target for this pathway (Kurz et al., 2002). Cullins are a common subunit of Roc1/Rbx1/Hrt1-Skp1-cullin/Cdc53-F box (SCF) complexes, which regulate the stability of various proteins involved in cell cycle control and signal transduction, including $p27^{Kip1}$, IκBα, and HIF1-α. (Deshaies, 1999; Karin and Ben-Neriah, 2000; Kondo and Kaelin, 2001; Ohh et al., 2002; Bloom and Pagano, 2003). Mechanistically, NEDDylation of cullins appears to regulate the E3 ubiquitin ligase activity of the SCF complex. Recent studies showed that NEDD8 modification facilitates the recruitment of the E2 ubiquitin-conjugating enzyme to the SCF complex and/or mediate dissociation of $p120^{Cand1}$, an inhibitor of cullins and SCF ligase activity (Kawakami et al., 2001; Liu et al., 2002; Wu et al., 2002). The ability of the Roc1/Rbx1/Hrt1 protein, a component of the SCF complexes, to promote NEDDylation of cullins suggested that it could act as an E3 NEDD8 ligase (Kamura et al., 1999; Gray et al., 2002; Morimoto et al., 2003).

The p53 tumor suppressor protein is a short-lived protein due to its rapid proteasomal degradation. Upon exposure of cells to various stress stimuli, levels of p53 rise as a consequence of reduced proteolytic degradation, and its function as a transcription factor is activated. This results in induction of a program of gene expression that promotes cell growth arrest and/or apoptosis (Lain, 2003; Woods and Lane, 2003). An im-

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portant regulator of the stability/activity of p53 is the Mdm2 oncogene product. Mdm2 directly interacts with p53, promoting its ubiquitination and degradation through the 26S proteasome (Bottger et al., 1997; Kubbutat et al., 1997; Haupt et al., 1997). The importance of this interaction was demonstrated when the lethality of *mdm2* null mice was rescued by simultaneous deletion of the *p53* gene (Jones et al., 1995; Luna et al., 1995). Furthermore, mice with a hypomorphic allele of *mdm2* exhibited increased p53 transcriptional activity and apoptotic function (Mendrysa et al., 2003). These data show that a key activity of Mdm2 in homeostatic tissues is to inhibit the growth-suppressing effects of p53. Biochemical studies showed that Mdm2 can act as an E3 ubiquitin ligase, and this activity depends on its RING finger domain at the C terminus of the protein (Honda et al., 1997; Honda and Yasuda, 2000; Fang et al., 2000; Midgley et al., 2000). Mdm2 can also regulate its own stability through an autoubiquitination activity, targeting itself for proteasomal degradation. There is now evidence that the "trans" and "auto" ubiquitin ligase activity of Mdm2 can be differentially regulated through its interaction with other proteins. The p14ARF tumor suppressor protein and the Mdm2 homolog Mdmx both interact with Mdm2 and can preferentially inhibit p53 ubiquitination or Mdm2 autoubiquitination, respectively (Xirodimas et al., 2001a; Stad et al., 2001). Other Mdm2-interacting proteins can also regulate the Mdm2-mediated degradation of p53, including TSG101, the retinoblastoma protein (pRb), the *c-Abl* protooncogene product, and p300 (Vousden, 2002; Michael and Oren, 2003). These data demonstrate that the E3 ubiquitin ligase activity of Mdm2 and the degradation pathway for p53 and Mdm2 are very tightly controlled in cells.

In this report, we demonstrate that Mdm2 promotes conjugation of NEDD8 to p53. Mdm2 is itself modified with NEDD8, and this reaction displays very similar characteristics to the autoubiquitination activity of Mdm2. The following experiments characterize this activity of Mdm2 and determine the biological significance of this modification as a mechanism of control of p53 and Mdm2 function.

Results

Mdm2 Is Conjugated to NEDD8 In Vivo

The only reported role for NEDD8 conjugation is to regulate the E3 ubiquitin ligase activity of SCF complexes through conjugation to cullin family member proteins (see Introduction). The Mdm2 protein is a well-characterized RING finger type E3 ubiquitin ligase that directs ubiquitination of the p53 tumor suppressor protein but also has a potent autoubiquitination activity. To determine if Mdm2 is a potential substrate for NEDD8 conjugation, we used a well-established approach where conjugation of different substrates to ubiquitin and ubiquitin-like molecules can be detected (Rodriguez et al., 1999; Xirodimas et al., 2001a).

H1299 cells (genetically negative for *p53*) were transfected with plasmids expressing either wild-type Mdm2 or Mdm2 mutants (Figure 1A) along with a vector expressing a Histidine-tagged (His_6) version of human NEDD8. Cells were lysed under strong denaturing conditions, His_6-NEDD8 conjugated species purified on Ni^{2+}-agarose beads, and samples analyzed by Western blotting using the 4B2 anti-Mdm2 antibody. While wild-type Mdm2 was conjugated to NEDD8, no conjugation was observed with any of the Mdm2 mutants used (Figure 1B, top panel). NEDD8 conjugation of Mdm2 appeared to depend on the cysteine residue 462 in the RING finger domain. Cysteine 462 (464 in human) has been previously characterized as one of the cysteines that coordinate zinc and is required for Mdm2 to promote its own ubiquitination as well as the ubiquitination of p53 (Fang et al., 2000; Honda and Yasuda, 2000).

Mdm2 Promotes NEDDylation of p53 In Vivo

The observation that Mdm2 could be conjugated to NEDD8 dependent on the Cys462, which is required for its ubiquitin ligase activity, prompted us to investigate the possibility that Mdm2 may act as an E3 NEDD8 ligase. One of the best-characterized substrates for Mdm2-mediated ubiquitination is the p53 tumor suppressor protein. To determine whether Mdm2 could promote conjugation of NEDD8 to p53, wild-type human p53 was expressed in H1299 cells along with the His_6-NEDD8 expression construct in the absence or presence of Mdm2. NEDDylated p53 was isolated on Ni^{2+}-NTA agarose and detected by Western blotting. Whole cell lysates were analyzed directly by Western blotting for determination of total p53 levels. NEDDylated p53 was detected in the absence of coexpressed Mdm2 (Figure 2A, lane 2); however, the amount of NEDDylated p53 was dramatically increased by Mdm2 coexperession (Figure 2A, lane 4). Treatment of cells with the proteasome inhibitor MG132 protected p53 protein from Mdm2-mediated downregulation and resulted in accumulation of p53 conjugated to NEDD8 (Figure 2A, lane 5). To further demonstrate that the immunoreactive p53 isolated by Ni^{2+}-NTA agarose is p53 conjugated to NEDD8, a recently described cysteine protease (NEDP1), which specifically cleaves NEDD8 molecules from conjugated substrates, was coexpressed (Mendoza et al., 2003). Whereas expression of p53 in the presence of His_6-NEDD8 and Mdm2 results in accumulation of modified p53 species, these species were not detected in the presence of NEDP1 (Figure 2B, lane 3). Expression of the NEDP1Cys, which is catalytically inactive, had no effect on p53 modification (Figure 2B, lane 4). The SSP3 SUMO-specific cysteine isopeptidase, which can reverse the SUMO-1 modification of p53 (Figure 2B, lane 9), had no effect on p53 modification with His_6-NEDD8 (Figure 2B, lane 5). The NEDP1 protease had no effect on Mdm2-mediated ubiquitination of p53, demonstrating the specificity of the protease for NEDD8 cleavage (Figure 2B, lane 13). The appearance of high molecular weight p53 species in our His_6-NEDD8 pull-downs suggested that other posttranslational modifications such as ubiquitin conjugation could simultaneously take place. To address this possibility, we used the p53-specific deubiquitinating enzyme HAUSP/USP7 (Everett et al., 1997; Li et al., 2002). As before, we transfected H1299 cells with p53, Mdm2, His_6-NEDD8 expression constructs, and, where indicated, increasing amounts of HAUSP or its catalytic inactive mutant, HAUSPCys. As shown in Figure 2C, HAUSP clearly rescued p53 from

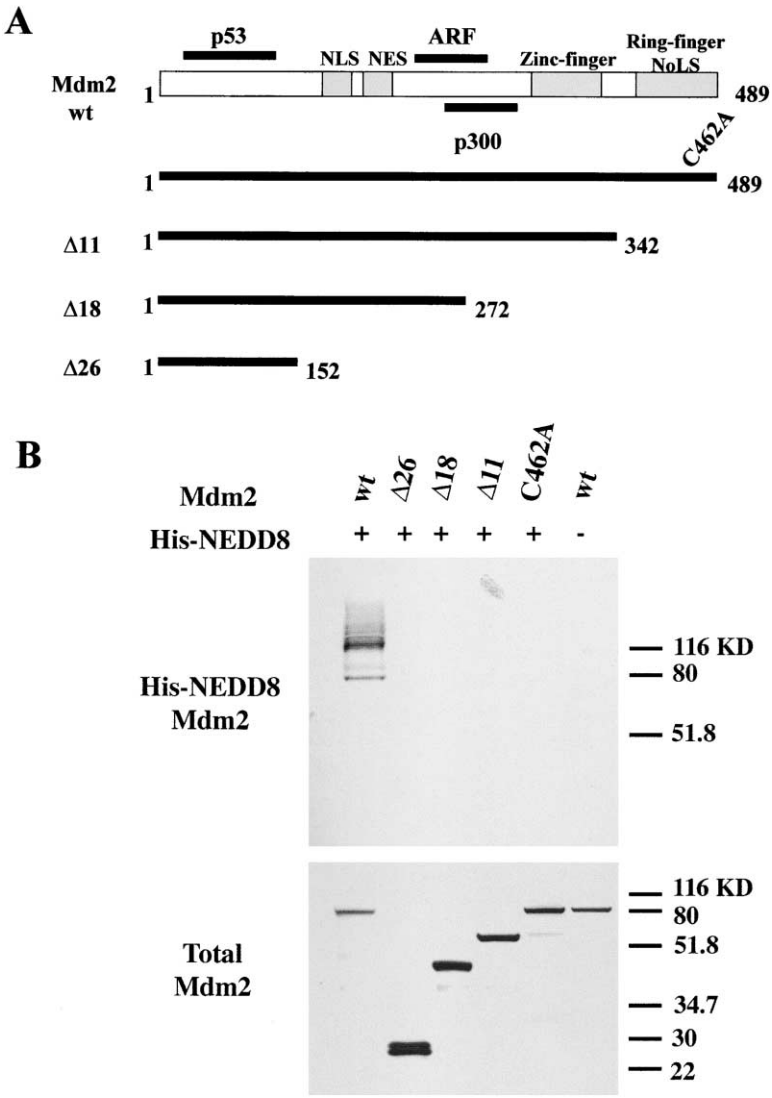


Figure 1. Mdm2 Is Conjugated to NEDD8 In Vivo

(A) Schematic representation of wild-type Mdm2 and Mdm2 mutants (Midgley et al., 2000).

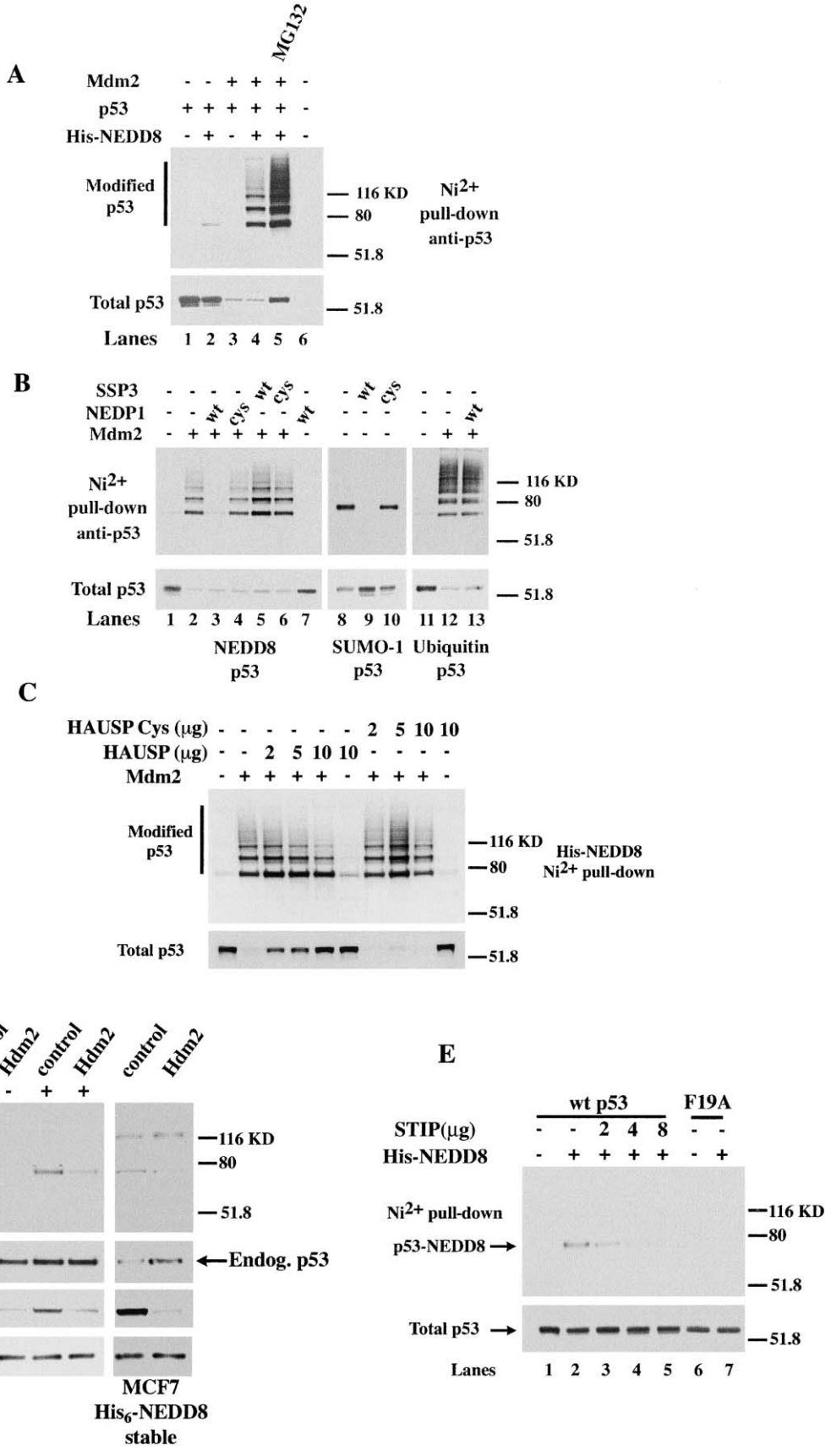
(B) H1299 cells were transfected as indicated with 5 μg of Mdm2 and 2 μg of His_6-NEDD8 expressing constructs. Posttransfection (36 hr), cells were lysed in guanidinium chloride, and His_6-NEDD8 conjugated species were purified on Ni^{2+}-NTA agarose as described in Experimental Procedures. NEDDylated Mdm2 (top panel) and total Mdm2 (bottom panel) were analyzed by Western blotting using the 4B2 monoclonal antibody.

Mdm2-mediated degradation (bottom panel). Under these conditions, HAUSP prevented the formation of high molecular weight p53 species but did not affect the more slowly migrating NEDD8 modified species, suggesting that NEDDylated p53 is also modified with ubiquitin.

Despite the overexpression of p53 and His_6-NEDD8, the NEDD8 conjugation activity observed in Figure 2A, lane 2, depends entirely on the endogenous NEDD8 conjugation machinery and endogenous E3 ligase(s). To determine whether this activity depends on the endogenous Hdm2, we performed the experiment described in Figure 2A in H1299 cells which had been transfected with either control or Hdm2 siRNA. As shown in Figure 2D, knockdown of endogenous Hdm2 resulted in decrease of NEDDylated p53. A similar experiment was performed in MCF7 cells that contain wild-type p53 and have been established to express His_6-NEDD8. Knockdown of Hdm2 resulted in upregulation of endogenous p53 levels and decrease of NEDDylated p53 (Figure 2D, right panel). Therefore, the ratio of NEDDylated p53 over total p53 protein is reduced upon knockdown of Hdm2 protein. To demonstrate that direct interaction of endogenous Hdm2 with p53 is required for p53 NEDDylation, we performed the experiment in H1299 cells in the presence of *s*uper *t*hioredoxin *i*nsert *p*rotein (STIP). This is a high-affinity (H)Mdm2 binding peptide in the p53 binding pocket, fused to thioredoxin, which disrupts the (H)Mdm2-p53 interaction (Bottger et al., 1997). Increasing amounts of transfected STIP reduced the amount of NEDDylated p53 detected (Figure 2E, lanes 3–5). Furthermore, a single point p53 mutant (F19A), which is previously shown to be deficient for Mdm2 binding (Liu et al., 2001; Xirodimas et al., 2001b), showed no NEDD8 modification under the same conditions. These data strongly suggest that NEDDylation of p53 depends on the expression of Hdm2 and that this reaction also requires interaction of p53 with endogenous Hdm2.

NEDDylation of p53 In Vitro

To address the action of Mdm2 as an E3 NEDD8 ligase for p53, we established an in vitro assay for NEDDylation of p53. Recombinant APP-BP1/Uba3 (E1), Ubc12 (E2), and NEDD8 were incubated with bacterially expressed


A
MG132
Mdm2
p53
His-NEDD8
Modified p53
116 KD
80
51.8
Ni2+ pull-down anti-p53
Total p53
51.8
Lanes 1 2 3 4 5 6
B
SSP3
NEDP1
Mdm2
wt
cys
Ni2+ pull-down anti-p53
116 KD
80
51.8
Total p53
51.8
Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13
NEDD8 p53
SUMO-1 p53
Ubiquitin p53
C
HAUSP Cys (μg)
HAUSP (μg)
Mdm2
Modified p53
116 KD
80
51.8
His-NEDD8 Ni2+ pull-down
Total p53
51.8
D
siRNA
control
Hdm2
His-NEDD8
Ni2+ pull-down
p53-NEDD8
116 KD
80
51.8
Total p53
Endog. p53
Endog. Hdm2
Actin
MCF7 His6-NEDD8 stable
E
wt p53
F19A
STIP(μg)
His-NEDD8
Ni2+ pull-down
p53-NEDD8
116 KD
80
51.8
Total p53
51.8
Lanes 1 2 3 4 5 6 7

p53 and Mdm2 in the presence of ATP, as described in Experimental Procedures. After incubation at 37°C for 2 hr, reactions were terminated and p53 species detected by Western blotting using the well-characterized DO-1 anti-p53 monoclonal antibody. Increasing amounts of added Ubc12 resulted in appearance of more slowly migrating p53 species whose molecular weights correspond to the addition of one or two NEDD8 molecules to p53. The appearance of these species was dependent on the addition of E1, E2, and NEDD8 (Figure 3A). Quantitation of the signals in each lane demonstrated a linear increase in p53 modification upon addition of increasing amounts of Ubc12 (Figure 3B). These data also show that modification of p53 with NEDD8 could occur in vitro in the absence of an E3 ligase. Addition of recombinant Mdm2 to reactions containing 100 ng of Ubc12 resulted in a moderate increase in NEDD8 modification of p53 (Figure 3C), while the same Mdm2 sample very efficiently promoted p53 ubiquitination. Quantitation of the modified species demonstrated that NEDD8 modification was stimulated by addition of increasing amounts of recombinant Mdm2 (Figure 3D).

Endogenous p53 and Mdm2 Are Modified with Endogenous NEDD8

To determine whether p53 and Mdm2 are subject to NEDD8 modification at endogenous expression levels, we performed immunoprecipitations using extracts from different cell lines with anti-p53 (DO-1), anti-Mdm2 (4B2), anti-NEDD8, or control IgG antibodies. DO-1 and 4B2 immunoprecipitates were analyzed by Western blotting using a polyclonal anti-NEDD8 antibody, whereas the reciprocal analysis was performed with the anti-NEDD8 immunoprecipitates. As shown in Figure 3E, a species of the predicted molecular weight for p53 modified with a single NEDD8 moiety was observed both in the DO-1 and anti-NEDD8 lane but not in the control IgG lanes. Similarly, for Mdm2, a specific band was detected above the 116 KD marker in the 4B2 and anti-NEDD8 immunoprecipitates but not in the control lanes (Figure 3F). This size corresponds to the predominant Mdm2 species detected in the overexpression experiment described in Figure 1B. These data strongly suggest that both endogenous p53 and Mdm2 are modified with NEDD8.

Specificity of Mdm2 as an E3 Ligase for p53

Mdm2 protein belongs to the RING finger family of ligases, and transfer of ubiquitin to itself and to p53 depends on the RING finger domain of the protein. To determine if the ability of Mdm2 to promote ubiquitin and NEDD8 conjugation of p53 was specific for these two molecules or could be extended to other ubiquitin-like molecules, p53 and Mdm2 were coexpressed with His_6 versions of ubiquitin and ubiquitin-like molecules such as NEDD8, SUMO-1, and ISG15. His_6-tagged purified species were analyzed for p53 by Western blotting as before. Mdm2 could clearly promote conjugation of ubiquitin and NEDD8 to p53 (Figure 3G, top panel, lanes 2 and 4) but had no effect on either SUMO-1 or ISG15 conjugation (Figure 3G, top panel, lanes 6 and 8). This is not a consequence of differences in the expression levels of the various ubiquitin-like proteins, as His_6-SUMO-1 and His_6-ISG15 are both expressed to higher levels than His_6-Ubiquitin or His_6-NEDD8 (data not shown). The decrease in SUMO-1 modification of p53 upon coexpression of Mdm2 is likely to be due to decrease of p53 protein levels (Figure 3G, lower panel, compare lanes 5 and 6).

Mdm2-Mediated NEDDylation of p53 Requires Lysines K370, K372, and K373 in the C Terminus of p53

Previous studies on Mdm2-mediated ubiquitination of p53 identified six lysines in the C terminus of p53 (K370, K372, K373, K381, K382, and K386), which are required for efficient Mdm2-mediated ubiquitination and proteasomal degradation of p53 (Nakamura et al., 2000; Rodriguez et al., 2000). To determine the lysines required for Mdm2-mediated NEDDylation of p53, three different sets of p53 lysine mutants were employed: the 3NKR (K370R, K372R, K373R), the 3CKR (K381R, K382R, K386R), and the 6KR (with all six lysines mutated into arginines). These mutants were either expressed alone or coexpressed with Mdm2 in the presence of either His_6-ubiquitin or His_6-NEDD8. Comparison of Mdm2-mediated ubiquitination with NEDDylation of these p53 mutants identified key differences in the lysine requirements for these two pathways (Figure 4A). Consistent with previous reports, mutation of six lysines in the C terminus of p53 (6KR mutant) was necessary to abrogate Mdm2-mediated ubiquitination and degradation of p53. However, Mdm2-mediated NEDDylation of p53 appeared to depend on lysines K370, K372, and K373, as NEDDylation of the 3NKR mutant was substantially reduced (Figure 4A). Importantly, like Mdm2-mediated ubiquitination of p53, NEDD8 conjugation was dependent on the direct interaction of p53 with Mdm2, as the F19A p53 mutant, which is deficient for Mdm2 binding (Liu et al., 2001; Xirodimas et al., 2001b), was also deficient for NEDDylation. Individual p53 lysine mutants

Figure 2. Mdm2 Promotes NEDDylation of p53 In Vivo

(A) H1299 cells were transfected with 1 μg of p53, 2 μg of Mdm2, and 2 μg of His_6-NEDD8 expression constructs as indicated. His_6-NEDDylated species were purified as before and analyzed by Western blotting with DO-1 anti-p53 monoclonal antibody (top panel). Total levels of p53 are shown on the bottom panel.

(B) H1299 cells were transfected as before, and, where indicated, 5 μg of NEDD8-specific protease NEDP1, SSP3 SUMO protease, or 2 μg of His_6-NEDD8, His_6-SUMO-1, His_6-Ubiquitin expression constructs were included.

(C) H1299 cells were transfected as above, and, where indicated, the p53-specific deubiquitinating enzyme HAUSP and its catalytic inactive mutant (HAUSP Cys) expression constructs were cotransfected. Modified p53 (top panel) and total p53 (bottom panel) were detected with the DO-1 anti-p53 antibody.

(D) H1299 cells were first transfected with control or Hdm2 siRNA (200 nM) and then with p53 and His_6-NEDD8 as described in Experimental Procedures. Cells were harvested and analyzed as before for NEDDylated and total p53.

(E) Wild-type p53 or F19A mutant was transfected in the absence or presence of His_6-NEDD8 and increasing amounts of STIP as indicated.

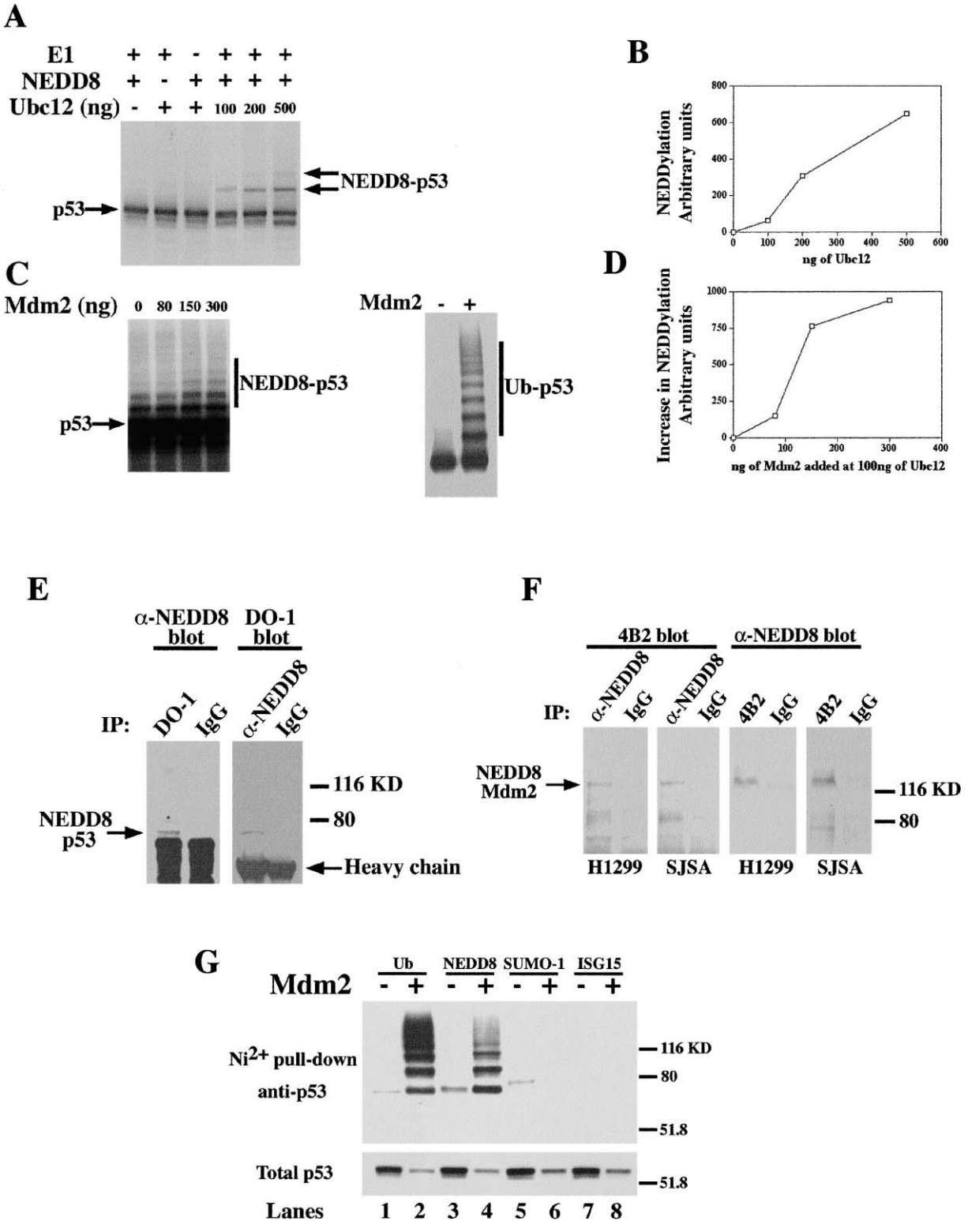


Figure 3. In Vitro NEDDylation of p53

(A) Reactions were performed as described in Experimental Procedures. Samples were analyzed by Western blotting with DO-1 anti-p53 monoclonal antibody. (B) Signals in each lane (A) were quantitated and expressed as increase in arbitrary units over background (no added NEDD8 lane). (C) In vitro reactions were performed as in (A), using 100 ng of Ubc12 in each reaction and increasing amounts of bacterially expressed Mdm2. (D) Signals (in [C]) were quantitated and expressed as increase in arbitrary units over background (no Mdm2 added). Ubiquitination assay was performed as described in Experimental Procedures. Endogenous p53 and Mdm2 are modified with endogenous NEDD8. (E) Cells (5×10^7 COS-1) were harvested and lysed and immunoprecipitated as described in Experimental Procedures with anti-

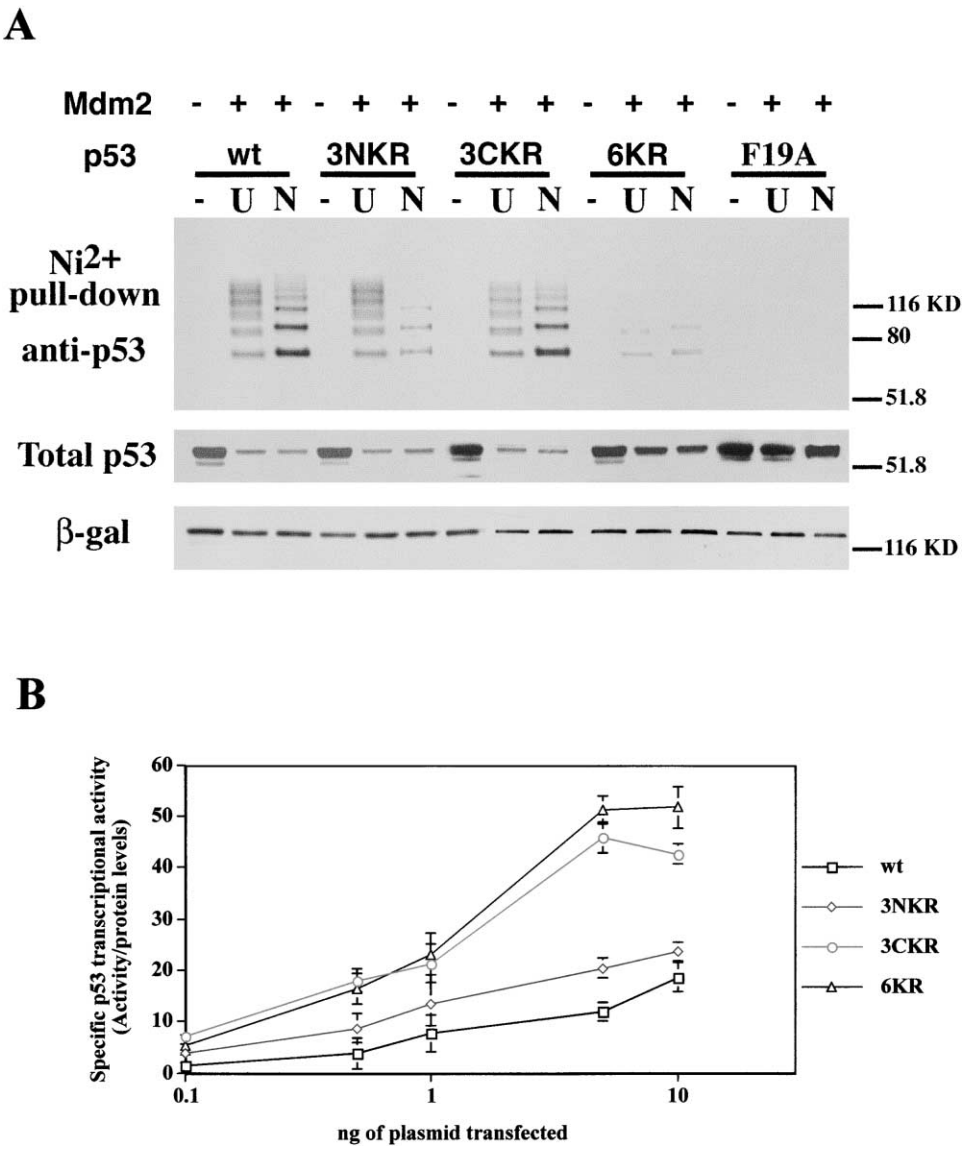


Figure 4. Mdm2-Mediated NEDDylation of p53 Requires Lysines K370, K372, and K373 in the C Terminus of p53

(A) H1299 cells were transfected with wild-type (wt) p53 or p53 lysine mutants, 3NKR (K370R, K372R, and K373R), 3CKR (K381R, K382R, and K386R), or 6KR (all six lysines mutated into arginine). Where indicated, 2 μg of Mdm2 and 2 μg of His_6-ubiquitin (U) or His_6-NEDD8 (N) were cotransfected. Ni^{2+}-agarose eluates and cell extracts were analyzed by Western blotting using the DO-1 anti-p53 antibody (top and middle panels). Equal transfection efficiency and loading are demonstrated by β-gal expression (lower panel, 1 μg of DNA was cotransfected).

(B) Specific transcriptional activity of wild-type p53 and p53 lysine mutants. Increasing amounts of p53 expression constructs were transfected in H1299 cells. Transcriptional activity was measured 24 hr posttransfection as described in Experimental Procedures. The same cell extracts were used to monitor the stability of wild-type p53 and p53 lysine mutants by two-site ELISA as described before (Xirodimas et al., 2001a). The presented data is the ratio of the transcriptional activity to protein levels (specific activity). Renilla was used to normalize the values in both assays.

were also used, but no differences were observed in Mdm2-mediated ubiquitination or NEDDylation of these mutants (data not shown).

Previous analysis of p53 lysine mutants in the C terminus of p53 showed that they exhibit higher transcriptional activity than wild-type p53 (Rodriguez et al., 2000).

NEDD8 polyclonal, DO-1 anti-p53 monoclonal antibody, or IgG control. Immunoprecipitates were analyzed by Western blotting with anti-NEDD8polyclonal antibody or DO-1 anti-p53 as indicated. (F) Cells (5×10^7 H1299) or SJSA were lysed as before and used for immunoprecipitations with anti-NEDD8, 4B2 anti-Mdm2, or IgG control antibodies. Immunoprecipitates were Western blotted with 4B2 or anti-NEDD8 antibodies as indicated. (G) Specificity of Mdm2 as an E3 ligase. H1299 cells were transfected with 1 μg of wild-type p53, 2 μg of Mdm2, and 2 μg of each of the His_6-tagged expression constructs. After 36 hr, His_6-tagged conjugated species were purified as before and analyzed by Western blotting using the DO-1 anti-p53 antibody (top panel). The lower panel shows total levels of p53.

The observed differences in the lysine requirements between ubiquitination and NEDDylation of p53 prompted us to reexamine the transcriptional activity of these mutants. H1299 cells were transfected with the indicated amounts of DNA for each p53 construct along with PG13-luc plasmid, in which a p53 response element drives the expression of luciferase. The same cell extracts were also analyzed by two-site ELISA to determine the amount of p53 protein in the same experiment. The ratio of the transcriptional activity to the protein levels of wild-type p53 and the p53 mutants will indicate the specific activities of the proteins and whether these mutations affect the intrinsic transcriptional activity of p53. As shown in Figure 4B, all p53 mutants tested displayed an increased specific transcriptional activity compared with wild-type p53. However, whereas the 6KR and 3CKR mutant were very similar, the 3NKR mutant showed an intermediate phenotype between wild-type p53 and the 6KR/3CKR mutants. The increased transcriptional activity of the 3NKR mutant, compared to wild-type protein, suggests that NEDD8 modification of p53 inhibits its transcriptional activity. This comparison also differentiates the role of these two sets of lysine residues in the control of the intrinsic transcriptional activity of p53.

Mutation of a E1-NEDD8 Component (APP-BP1) Increases the Transcriptional Activity of p53 and Facilitates the Inhibitory Effect of Mdm2 on p53-Dependent Transcription

To test the biological significance of NEDD8 conjugation of p53, we used a previously described cell line (CHO-TS-41) that contains a temperature-sensitive mutation in the *APP-BP1* gene (Cernac et al., 1997). The gene product interacts with Uba3 to form a heterodimeric complex, which acts as the E1-activating enzyme for NEDD8. This biological system has been widely used to demonstrate the effect of NEDD8 conjugation pathway on the function of SCF E3 ubiquitin ligases (Chen et al., 2000; Querido et al., 2001; Ohh et al., 2002; Bloom et al., 2003). In our study, by growing these cells at the restrictive temperature (39°C), we could specifically study the effect of the NEDD8 conjugation pathway on p53 stability/activity. Indeed, as shown in Figure 5A (lanes 2 and 5), when the TS-41 cells were grown at 32°C, Mdm2 could efficiently promote conjugation of ubiquitin and NEDD8 to p53. However, at 39°C, NEDD8 conjugation of p53 was dramatically reduced (Figure 5A, compare lanes 5 and 11), while ubiquitination was not affected (Figure 5A, compare lanes 2 and 8). This suggests that the NEDD8 conjugation pathway does not regulate Mdm2-mediated ubiquitination of p53. Therefore, we used this system to determine the effect of NEDD8 conjugation on the transcriptional activity of p53. TS-41 and the wild-type CHO cells were transfected with wild-type p53 alone or with increasing amounts of Mdm2 expression construct along with the PG13-luc reporter plasmid. Cells were then either kept at 32°C or shifted to 39°C for a further 24 hr, and cell extracts were analyzed for p53 activity. Temperature shift increased the activity of p53 in the TS-41 cells, whereas no significant changes were observed in the wild-type CHO cells (Figure 5B). This is consistent with the idea that NEDD8 conjugation inhibits the transcriptional activity of p53. Furthermore, changes were observed in the ability of Mdm2 to inhibit the transcriptional activity of p53. In wild-type CHO cells, temperature shift abrogated the inhibitory effect of Mdm2 on the activity of p53. However, in TS-41 cells, Mdm2 appeared to suppress p53 activity more efficiently at the restrictive temperature (39°C) (Figure 5C). Cell extracts from a replica experiment were also used to monitor the expression levels of Mdm2 and p53 proteins (Figure 5D). Temperature shift to 39°C in the TS-41 cells appeared to increase both the levels of Mdm2 and p53 proteins. However, under these conditions, Mdm2 efficiently decreased the levels of p53. The opposite phenotype was observed in the wild-type CHO cell line. Temperature shift to 39°C decreased the levels of Mdm2, and p53 appeared resistant to Mdm2-mediated degradation. This is consistent with the observation that Mdm2 suppressive effect on p53 transcriptional activity was inhibited in the wild-type CHO cells at 39°C.

To determine whether the effects on p53 transcriptional activity in the TS-41 cells were due to mutation in the *APP-BP1* gene, we performed the transcriptional assays described above in the presence of coexpressed wild-type APP-BP1. A titration of increasing amounts of transfected APP-BP1 construct showed that, at low concentrations, wild-type APP-BP1 could rescue the p53 transcriptional phenotype observed at 39°C (Figure 5E). As shown before, Mdm2 at the restrictive temperature dramatically reduced the transcriptional activity of p53. However, coexpression of wild-type APP-BP1 reversed the effect of Mdm2 on p53 function, restoring the phenotype observed at 32°C. Expression of APP-BP1 alone reduced the activity of p53, restoring the phenotype observed at 32°C. These data strongly suggest that the differences observed in p53 activity and Mdm2 function at 39°C were due to mutation of the E1 NEDD8 component APP-BP1.

The NEDD8 Conjugation Pathway Requires Lysines K370, K372, and K373 in p53 to Regulate p53 Transcriptional Activity

The transcriptional data obtained using the TS-41 cell line demonstrated that NEDD8 conjugation controls both p53 and Mdm2 function. Our mutational analysis on p53 demonstrated that lysines K370, K372, and K373 in p53 are required for Mdm2-mediated NEDDylation of the protein. This p53 mutant, in combination with the TS-41 cell line where the NEDD8 conjugation pathway can be selectively switched off, provides the tools to (1) demonstrate that the increased transcriptional activity of p53 at the restrictive temperature in the TS-41 cells is due to lack of NEDDylation of p53 and not to lack of NEDDylation of cullins or other yet unidentified NEDD8 substrates; and (2) to differentiate the effect of NEDD8 modification on the transcriptional activity of p53 and the ability of Mdm2 to suppress p53-dependent transcription. Wild-type p53 and the 3NKR p53 mutant were expressed in TS-41 or wild-type CHO cells. As before, cells were kept at 32°C or shifted to 39°C, and p53 transcriptional activity was measured 24 hr later. As shown before, a shift of the TS-41 cells to the nonpermissive temperature increased the transcriptional activity of wild-type p53. However, the 3NKR mutant, which is

resistant to Mdm2-mediated NEDDylation, displayed an increased transcriptional activity at the permissive temperature, and shift to 39°C did not significantly increase its activity (Figure 6A). This strongly suggests that the increase in p53 activity in the TS-41 cells at the restrictive temperature is due to lack of p53 NEDDylation. We also used the 3NKR mutant to investigate the role of the NEDD8 conjugation pathway in the activity of Mdm2 as a suppressor of p53 activity. Wild-type p53 and the 3NKR mutant were expressed either alone or with increasing amounts of Mdm2 in the TS-41 cells at the permissive and restrictive temperature. The 3NKR mutant appeared to resist transcriptional repression by Mdm2 compared with wild-type p53 at 32°C in TS-41 cells (Figure 6B). However, at 39°C, Mdm2 could efficiently suppress the activity of the 3NKR mutant in a very similar manner to that of wild-type p53 suppression. These data provide evidence that the NEDD8 conjugation pathway also controls the ability of Mdm2 to suppress p53 function.

Differential Regulation of p53 Ubiquitination and NEDDylation by UV-Induced DNA Damage

The stability of the p53 protein is subject to regulation by a variety of stress stimuli, including DNA damage induced by γ rays (IR) or UV light. To determine the effect of DNA damage on modification of endogenous p53 with NEDD8 or ubiquitin, we used MCF7 cells, which contain wild-type p53 and had been established to express either His_6-NEDD8 or His_6-ubiquitin. As control, we used parental MCF7 cells, which do not express tagged NEDD8 or ubiquitin. Cells were irradiated with UV, and, 4 or 8 hr postirradiation, His_6-NEDDyalted or His_6-ubiquitinated species were purified as before and analyzed for p53 modification by Western blotting. As shown in Figure 7A, endogenous p53 is modified both with ubiquitin and NEDD8 (UV, 0 hr). UV treatment resulted in increased p53 protein levels in all three systems. Ubiquitin modification of p53 was inhibited 4 hr post-UV treatment and sustained 8 hr after irradiation. NEDD8 modification of p53 was transiently increased 4 hr after UV irradiation but could not be detected by 8 hr post-UV irradiation. NEDD8 modification and ubiquitination of p53 therefore displayed different kinetics in response to UV irradiation. These data show that p53 NEDDylation, like ubiquitination, is subject to DNA damage response signals controlling p53 activity, but these p53 modifications are differentially controlled.

Discussion

Mdm2 Promotes Ubiquitin and NEDD8 Modification of p53: A Common Feature for RING Finger Ligases?

In this paper, we demonstrate that the Mdm2 RING finger E3-ubiquitin ligase is NEDDylated and promotes conjugation of NEDD8 to p53. While in vitro Mdm2 could produce a moderate stimulation on NEDD8 modification of p53, the in vivo data demonstrate that p53 NEDDylation depends on Hdm2 expression and direct binding of p53 to H(M)dm2. Also, the critical cysteine C462 in the RING finger domain of the protein, which is required for the E3 ubiquitin ligase activity of Mdm2, is also required for Mdm2-dependent NEDDylation. This evidence demonstrates a key role for Mdm2 in p53 NEDDylation and strongly suggests that Mdm2 acts as an E3 NEDD8 ligase.

Using p53 as a substrate, Mdm2 was limited to the conjugation of ubiquitin and NEDD8 but not SUMO-1 or ISG15. In the other characterized system for NEDD8 conjugation, Rbx1, the RING finger component of the SCF complex, which is required for ubiquitin ligase activity, can also act an E3 NEDD8 ligase for cullins (see Introduction). These observations raise the possibility that the dual usage of ubiquitin and NEDD8 could be a common feature of the RING finger type of E3 ligases.

Modification with NEDD8 Inhibits the Transcriptional Activity of p53

The data presented in this study demonstrate a role for NEDD8 conjugation of p53 as a mechanism of control of p53 transcriptional activity. Mdm2-mediated NEDDylation of p53 requires three lysines (K370, K372, and K373) at the C terminus of the protein (3NKR p53 mutant). Earlier reports demonstrated that lysines K370, K372, K373, K381, K382, and K386, located in the C terminus of p53, are required for Mdm2-mediated ubiquitination and proteasomal degradation of p53. This group includes the lysines that are required for NEDD8 modification, but the clear difference in the lysine requirement for NEDD8 and ubiquitin conjugation of p53 indicates the differential specificity of Mdm2 for these two conjugation pathways. The 3NKR mutant displayed an increased transcriptional activity compared to wild-type p53 in H1299 cells, suggesting that NEDDylation of p53 inhibits its function.

The use of the TS-41 CHO cell line provided a good biological system to assess the biological significance of the NEDD8 conjugation pathway in the transcriptional activity and stability of p53. At the restrictive temperature where the NEDD8 conjugation pathway is impaired (Figure 5A), the transcriptional activity of p53 was increased, and the levels of p53 protein were moderately elevated (Figure 5B, 5D no Mdm2 cotransfection lane), consistent with a suppressive role for NEDD8 conjugation for p53 activity. The 3NKR p53 mutant, which is deficient for Mdm2-mediated NEDDylation, displayed an increased transcriptional activity compared to wild-type p53 at 32°C with no dramatic increase upon temperature shift. This demonstrates that direct NEDDylation of p53 can control p53 transcriptional activity.

The use of proteases that specifically remove ubiquitin, NEDD8, or SUMO-1 provided a powerful tool to investigate the different modifications of p53. The specific deubiquitinating p53 enzyme HAUSP rescued p53 from Mdm2-mediated degradation. Under these conditions, HAUSP prevented the formation of high molecular weight p53 species in the NEDD8 Ni^{2+} pull-down, suggesting that these species are ubiquitin conjugates and that p53 could be simultaneously modified with NEDD8 and ubiquitin. However, while HAUSP dramatically increased p53 protein levels in the presence of Mdm2 (Figure 2C, bottom panel), there was no significant increase in the p53 levels upon expression of the deNEDDylating enzyme NEDP1 (Figure 2B, lanes 2 and 3). This suggests that NEDD8 modification of p53 does not

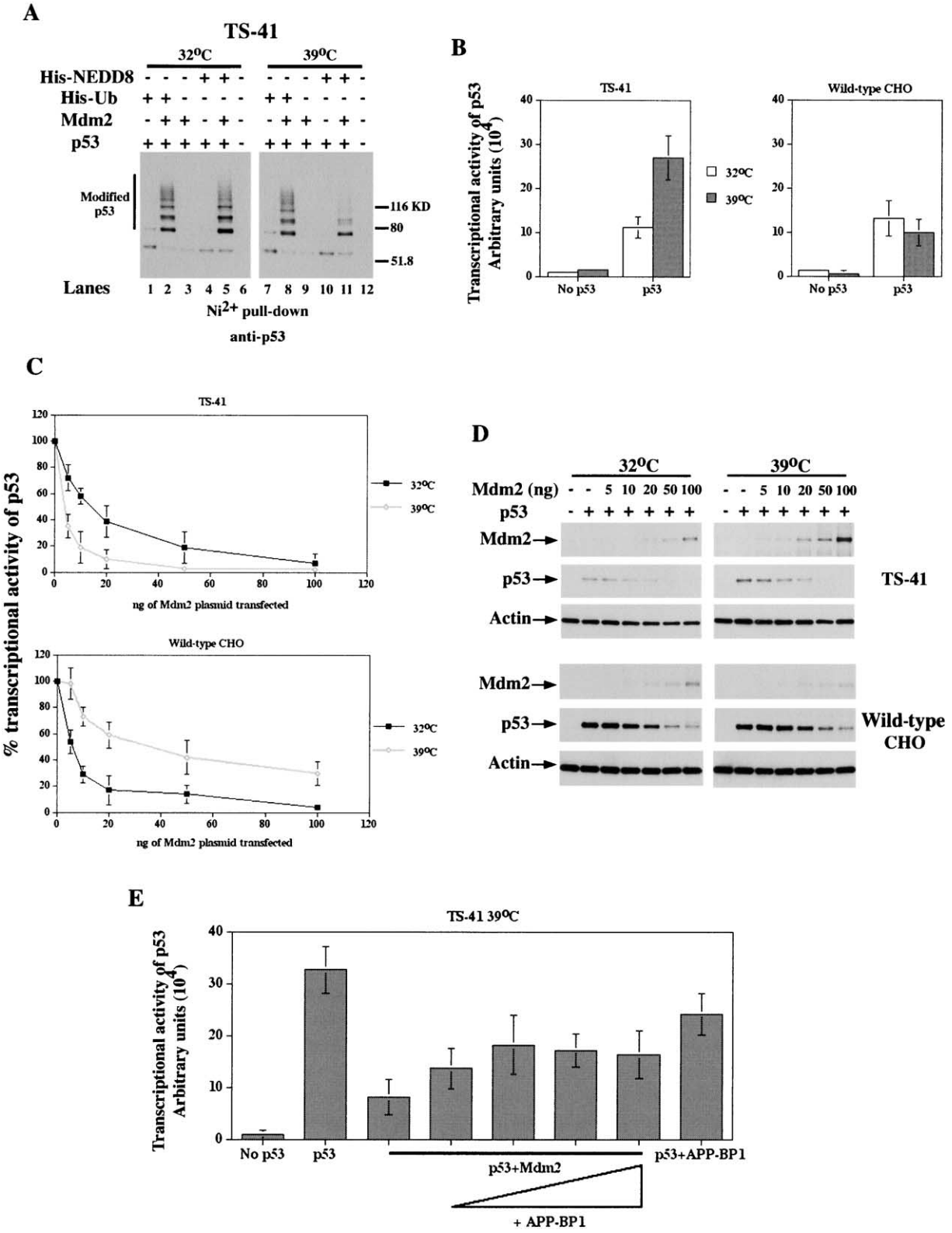


Figure 5. The NEDD8 Pathway Controls the Function of p53 and Mdm2

(A) Mdm2-mediated NEDDylation of p53 is impaired at 39°C in TS-41 cells. TS-41 cells, where indicated, were transfected with 1 μg of p53, 2 μg of Mdm2, 2 μg of His_6-ubiquitin (His_6-Ub), or His_6-NEDD8 expression constructs. Cells were either kept at 32°C or shifted to 39°C and 24 hr later His_6-ubiquitinated, or His_6-NEDDylated species were purified as described before. Samples were analyzed for p53 modification by Western blotting using the DO-1 monoclonal antibody. (B) Wild-type p53 expression construct (10 ng) was transfected either alone or with increasing amounts of Mdm2 (C) as indicated in TS-41 or wild-type CHO cells. The next day, cells were either kept at 32°C or shifted to 39°C,

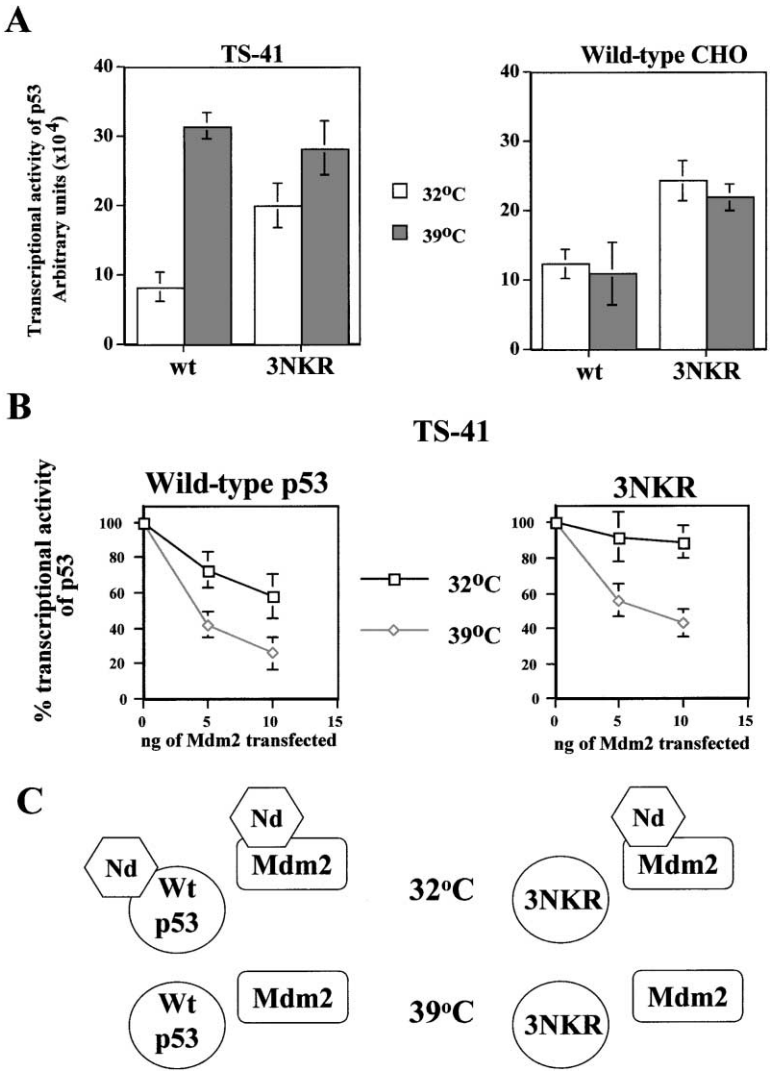


Figure 6. The NEDD8 Conjugation Pathway Requires Lysines K370, K372, and K373 in p53 to Control its Transcriptional Activity

(A) Wild-type p53 or 3NKR lysine mutant (10 ng) were transfected in TS-41 and wild-type CHO cells. Cells were either kept at 32°C or shifted to 39°C, and p53 transcriptional activity was measured 24 hr later.

(B) The NEDD8 conjugation pathway controls the suppressive effect of Mdm2 on p53 transcriptional activity. Wild-type p53 or 3NKR lysine mutant (10 ng) were transfected in TS-41 cells either alone or with increasing amounts of Mdm2 expression construct. Temperature shift and measurement of p53 transcriptional activity was performed as before. Values are expressed as percentage changes in activity.

(C) Schematic representation of the NEDD8 modification status for p53 and Mdm2 in the above experiment.

have a significant effect on Mdm2-mediated degradation of p53.

NEDD8 Conjugation Pathway Inhibits the Suppressive Role of Mdm2 on p53 Function

The use of the TS-41 CHO cell line also demonstrated the role of NEDD8 modification on Mdm2 function as an inhibitor of p53 transcriptional activity. In wild-type CHO cells, Mdm2's ability to suppress p53 function was inhibited upon temperature shift to 39°C (Figure 5C). This could be due to heat stress, which may block Mdm2-mediated suppression of p53. However, this effect was clearly reversed in the TS-41 cells at the restrictive temperature, and Mdm2 appeared to be a better inhibitor of the transcriptional activity of p53 (Figure 5C). The observation that temperature shift also resulted in increased Mdm2 protein levels in TS-41 cells (Figure 5D) may explain Mdm2's increased capacity to suppress p53 function under these conditions. These data suggest that NEDD8 conjugation also suppresses the inhibitory role of Mdm2 on p53 function.

The use of the 3NKR mutant in the TS-41 cell line provided a good approach to differentiate the role of NEDD8 modification in the activity of p53 and Mdm2. This mutant is impaired for Mdm2-mediated NEDDylation, so we could specifically address the role of NEDD8 conjugation on the ability of Mdm2 to inhibit p53 function. At conditions where the NEDD8 conjugation path-

and p53 transcriptional activity was measured 24 hr later. (D) Cells from a replica experiment were lysed with 2 × SDS buffer and analyzed by Western blotting for p53 and Mdm2 protein levels. Loading was monitored by actin. It has to be noted that, for each protein, the same exposure time was employed, but different exposure times were used between different cell lines. Therefore, no comparison of protein levels should be made between different cell lines. (E) Expression of wild-type APP-BP1 rescues the phenotype of the TS-41 cells at 39°C. TS-41 cells were transfected with 10 ng of p53; 10 ng of Mdm2; and 20, 50, 100, and 200 ng of APP-BP1 expression constructs as indicated. Cells were shifted to 39°C, and p53 transcriptional activity was measured 24 hr later. When p53 and APP-BP1 were coexpressed, 200 ng of APP-BP1 expression construct was used.

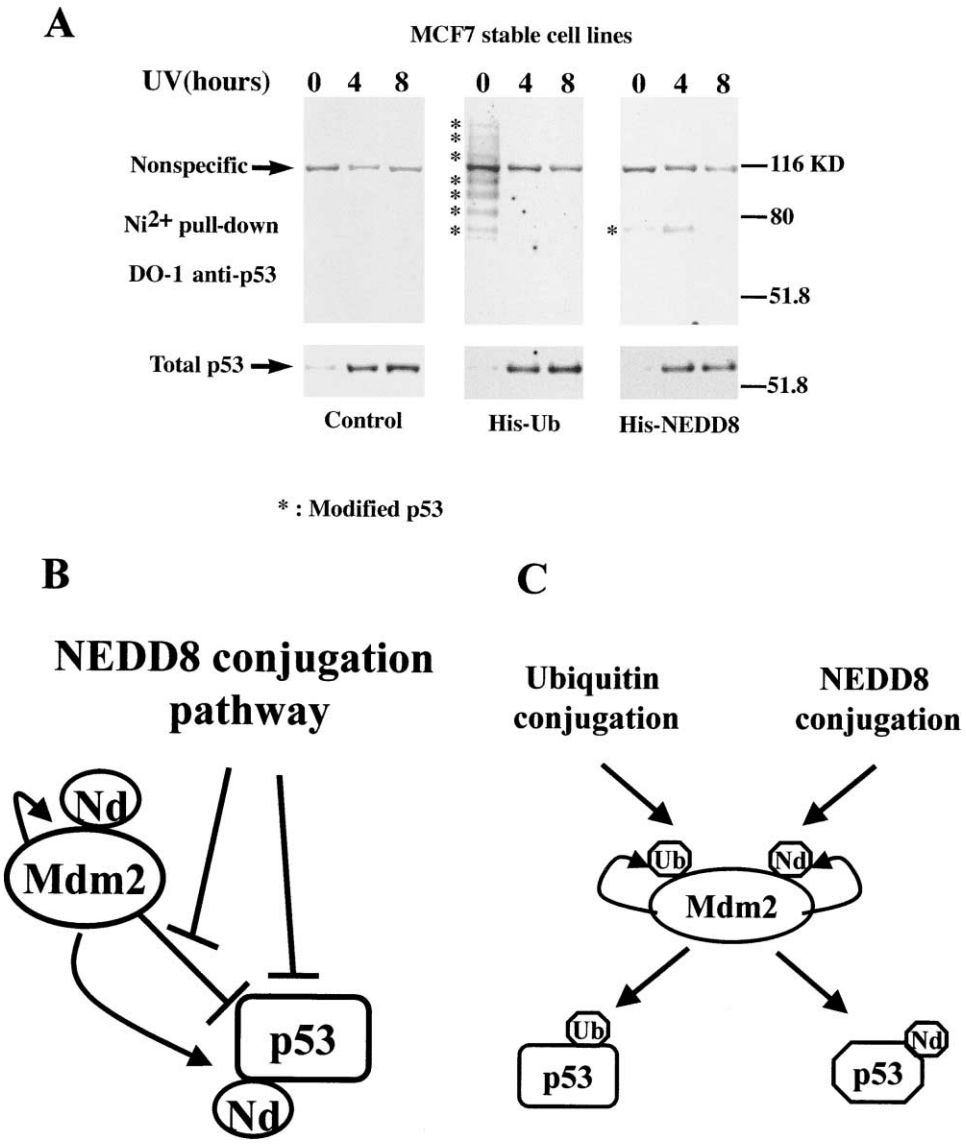


Figure 7. Differential Regulation of Ubiquitin and NEDD8 Modification of p53 upon UV-Induced DNA Damage

(A) Control MCF7 or MCF7 cells stably expressing either His_6-ubiquitin or His_6-NEDD8 were irradiated with UV (30 joules/m^2). At the indicated time points, His_6-tagged conjugates were purified as before, and eluates were used for Western blotting with the DO-1 anti-p53 antibody. Modification of endogenous p53 with ubiquitin or NEDD8 is shown on the top panel. The bottom panel shows total levels of endogenous p53 in that experiment.

(B) A model for the role of the NEDD8 conjugation pathway as a negative regulator of p53 and Mdm2 function. Mdm2 protein promotes NEDDylation (Nd) of p53 and itself. The NEDD8 conjugation pathway inhibits both the transcriptional activity of p53 and the suppressive effect of Mdm2 on p53 function. In this way, Mdm2 negatively controls the function of p53 and itself through the same pathway.

(C) Mdm2 at the center of ubiquitin (Ub) and NEDD8 (Nd) conjugation pathways. Diversity for the regulation of Mdm2 and p53 function.

way is active (32°C), the NEDDylation-deficient p53 mutant (3NKR) was resistant to Mdm2-mediated suppression. This resistance was clearly inhibited at the restrictive temperature (39°C), where the NEDD8 conjugation pathway is switched off, thus demonstrating that the NEDD8 pathway also controls the inhibitory effect of Mdm2 on p53 function.

The data support a model where both p53 and Mdm2 are subjects of a negative regulation by the NEDD8 conjugation pathway (Figure 7B). Mdm2 protein by promoting NEDD8 modification negatively regulates the function of p53 and itself through the same pathway. This is also the role of ubiquitin conjugation as it negatively regulates p53 and Mdm2 proteins by targeting them for proteasomal degradation.

Previous reports have suggested a link between the NEDD8 conjugation pathway and p53 function. The COP9 signalosome (CSN) was shown to phosphorylate p53 and control its proteasomal degradation by the E6/AP and Mdm2 pathway (Bech-Otschir et al., 2001). Mice, in which the genes encoding for the COP9 signalosome subunits 2 and 3 (*Csn2*, *Csn3*) were disrupted, showed deficiencies in cell proliferation and increase in p53 and cyclin E protein levels (Lykke-Andersen et al., 2003; Yan

et al., 2003). Furthermore, the NEDD8 conjugation pathway was implicated in p53 degradation by adenovirus E4orf6 and E1B55K proteins (Querido et al., 2001). The discovery that Mdm2 E3 ubiquitin ligase can also stimulate the conjugation of p53 and itself to NEDD8 provides direct evidence for a functional link between the NEDD8 conjugation pathway and regulation of p53 and Mdm2 function.

Mdm2 at the Center of Ubiquitin and NEDD8 Conjugation Pathways: Diversity for RING Finger Ligases as Regulators of Protein Function

The discovery that Mdm2 can use both the ubiquitin and NEDD8 conjugation pathways positions it at the center of these two pathways (Figure 7C). However, the observation that Mdm2-mediated ubiquitination of p53 was not affected at conditions where Mdm2-mediated NEDDylation of p53 could be inhibited (39°C in TS-41 cells, Figure 5A), suggests the independent regulation of the ability of Mdm2 to promote ubiquitin and NEDD8 conjugation to p53. It also indicates that the E3 ubiquitin ligase activity of Mdm2 is not controlled through NEDDylation of cullins, the other well-characterized substrate for NEDD8 modification. The differential pattern of ubiquitin and NEDD8 modification of endogenous p53 after UV-induced DNA damage shows that NEDD8 modification is part of p53 activity control and further supports the idea that these two conjugation pathways are differentially regulated. The dual role of Mdm2 in the ubiquitin and NEDD8 conjugation pathways suggests a highly controlled system for Mdm2 function and also indicates the diversity of E3 ligases as regulators of protein function.

Experimental Procedures

Antibodies and Chemicals

p53 and Mdm2 were detected using the DO-1 and 4B2 mouse monoclonal antibodies, respectively (Stephen et al., 1995; Chen et al., 1993). The anti-NEDD8 rabbit polyclonal antibody and NEDD8 protein were purchased from Alexis Biochemicals. Anti-β-gal and anti-actin mouse monoclonal antibodies were purchased from Oncogene. MG132 was purchased from Calbiochem and iodoacetamide from Sigma. siRNAs were purchased from Dharmacon.

Cell Culture and Transfections

All cell lines were grown in medium with 10% fetal bovine serum. H1299 cells were grown in RPMI, CHO, and TS-41 cells in F12 CHO cell line medium, and COS-1 in DMEM. All transfections were performed using FuGENE 6 according to manufacturer's instructions, except for the experiments in H1299 cells, where the calcium phosphate method was used in 10 cm tissue culture plates. For the siRNA experiment, 2×10^5 H1299 cells were plated in 6-well dishes and transfected with siRNA (200 nM) by oligofectamine. Next-day cells were reseeded, transfected the day after with p53 (0.5 μg) and His_6-NEDD8 (1 μg) by using FuGENE 6, and harvested 24 hr later.

Plasmids

Human NEDD8 and ISG15 were amplified from fetal human liver cDNA and cloned in frame with His_6 in pcDNA3 vector. Ubc12 cDNA was received as an IMAGE clone from Research Genetics/Incyte Genomics (ID 3344837). The Ubc12 cDNA was amplified and cloned in the pGEX-2TK vector (Pharmacia). All sequences were confirmed by automated sequencing. The APP-BP1 construct (Chen et al., 2000) was kindly provided by Dr. Rachel Neve. All other plasmids are described elsewhere (Rodriguez et al., 1999; Xirodimas et al., 2001a, 2002).

Purification of His_6-Tagged Conjugates

Posttransfection cells (36 hr) were harvested, and His_6-tagged conjugates were purified as described before (Rogriguez et al., 1999; Xirodimas et al., 2001a). Twenty percent of cells were used for NP-40 lysis, and Western blotting was performed as described in Xirodimas et al. (2001a).

In Vitro NEDDylation Assays

SF9 cells were infected with viruses kindly provided by Dr Kazuhiro Iwai, expressing His-tagged APP-BP1 and T7-tagged Uba3. The E1 NEDD8 activating heterodimer enzyme was purified from extracts using Ni^{2+}-NTA agarose. GST-Ubc12 was expressed in bacteria, and, after purification through glutathione sepharose beads, the GST was cleaved with thrombin. Thrombin and any free GST were removed with benzamidine and glutathionine sepharose beads, respectively. Mdm2 and p53 proteins were expressed in bacteria. Mdm2 was purified from inclusion bodies, whereas p53 in the soluble fraction was partially purified using a heparin column (Pharmacia). p53 (40 ng) was incubated in a 15 μl reaction including an ATP regenerating system (50 mM Tris [pH 7.5], 5 mM $MgCl_2$, 2 mM ATP, 10 mM creatine phosphate, 3.5 U/ml creatine kinase, and 0.6 U/ml inorganic pyrophosphatase), 120 ng of NEDD8 activating enzyme (APP-BP1/Uba3), the indicated amounts of Ubc12 and Mdm2 proteins, and 5 μg of NEDD8. The reactions were incubated at 37°C for 2 hr and then terminated with 2 × SDS sample buffer containing β-mercaptoethanol. The samples were fractionated in 10% Novex gels and analyzed by Western blotting for p53 using the DO-1 monoclonal antibody. For the ubiquitination assay, 50 ng of E1 activating enzyme, 200 ng of UbcH5a, and 150 ng of Mdm2 were used in the NEDDylation reaction buffer.

Immunoprecipitations

Cells (5×10^7 COS-1, H1299, SJSA) were lysed in 750 μl of PBS containing 1% NP-40, 1% SDS, 5 mM EDTA, 10 mM iodoacetamide, and Protease Inhibitor Cocktail (Roche). After lysis, extracts were incubated at 60°C for 10 min, and 10 mM of DTT was added, diluted 10 times with PBS-NP-40, and precleared with 200 μl of protein G beads (50% slurry) for 45 min at 4°C. For each immunoprecipitation, 5 μg of DO-1 or IgG and 10 μl of 4B2 ascites or anti-NEDD8 serum were used, and samples were incubated for 3 hr at 4°C. Fifty percent slurry protein G beads (20 μl) were then added, and samples were rotated at 4°C for 45 min. Beads were washed five times with 500 μl of PBS-NP-40, and 20 μl of 2 × SDS loading buffer was added. Immunoprecipitates were analyzed by Western blotting as indicated.

Transcriptional and Temperature Shift Experiments

In a 24-well plate, 45,000 cells/well were seeded and transfected using FuGENE 6. p53 (10 ng), 50 ng of PG-13-luciferase, and 1 ng of Renilla expression constructs were used in each transfection. The total amount of transfected plasmid was made up to 300 ng with empty pcDNA3 vector. The cells were incubated at 32°C overnight and were either shifted to 39°C or kept at 32°C for a further 24 hr. Cells were lysed with 1 × passive lysis buffer (Promega), and luciferase and Renilla activities were measured using the Berthold Microplate Luminometer LB 96V. Renilla values were used to control transfection efficiency and cell death.

Two-Site ELISA

Stability of p53 was monitored by two-site ELISA as described in Xirodimas et al. (2001a).

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