MOLECULAR AND CELLULAR BIOLOGY, Dec. 2007, p. 8284–8295 Vol. 27, No. 23
0270-7306/07/$08.00+0 doi:10.1128/MCB.00050-07

Ubiquitination and Degradation of Mutant p53▽

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Received 10 January 2007/Returned for modification 9 March 2007/Accepted 19 September 2007

While wild-type p53 is normally a rapidly degraded protein, mutant forms of p53 are stabilized and accumulate to high levels in tumor cells. In this study, we show that mutant and wild-type p53 proteins are ubiquitinated and degraded through overlapping but distinct pathways. While Mdm2 can drive the degradation of both mutant and wild-type p53, our data suggest that the ability of Mdm2 to function as a ubiquitin ligase is less important in the degradation of mutant p53, which is heavily ubiquitinated in an Mdm2-independent manner. Our initial attempts to identify ubiquitin ligases that are responsible for the ubiquitination of mutant p53 have suggested a role for the chaperone-associated ubiquitin ligase CHIP (C terminus of Hsc70-interacting protein), although other unidentified ubiquitin ligases also appear to contribute. The contribution of Mdm2 to the degradation of mutant p53 may reflect the ability of Mdm2 to deliver the ubiquitinated mutant p53 to the proteasome.

The p53 tumor suppressor gene encodes a protein that accumulates in the cells in response to a variety of stresses, activating a number of responses that include cell cycle arrest, apoptosis, and DNA repair. p53 functions as a transcription factor, activating the expression of various target genes that mediate the p53 responses (59). Structurally, p53 contains N-terminal transactivation domains, a proline-rich regulatory domain, a central sequence-specific DNA binding domain (DBD), and a C-terminal regulatory region containing nuclear import and export sequences as well as the oligomerization domain (21) (see Fig. 1).

In normal cells, p53 is kept inactive through a number of mechanisms, including the activity of the oncoprotein Mdm2, a key negative regulator of p53 (1, 38, 58). p53 transcriptionally activates the expression of Mdm2 in a negative feedback loop (65). The critical role of Mdm2 in inhibiting p53 is best illustrated by results from studies of mice, in which embryonic lethality caused by the loss of mdm2 was completely eliminated by the simultaneous deletion of p53 (26). Mdm2 can inhibit the transcriptional activity of p53 by binding directly to the N-terminal transactivation domain (41, 43). However, Mdm2 also functions as an E3 ligase, covalently attaching ubiquitin molecules to p53 (16, 24), which leads to both the export of p53 to the cytoplasm (3, 8, 18, 20, 33) and proteasomal degradation (22, 27). Although Mdm2 plays an important role in regulating p53 stability, a number of other E3 ligases have recently been identified that can promote the degradation of p53 independently of Mdm2. These include Pirh2 (32), Cop1 (14), TOPORS (46, 62), ARF-BP1 (10), Synoviolin (66), Carps (54), and CHIP (C terminus of Hsp70-interacting protein) (15).

The interaction between p53 and Mdm2 is necessary for the ubiquitination and degradation of p53 by Mdm2 (27, 38, 40). A highly conserved region in the N terminus of p53 (box I, amino acids [aa] 13 to 18) interacts with a hydrophobic binding pocket in the N-terminal domain of Mdm2 (aa 25 to 109), and small-molecule inhibitors of this interaction stabilize p53 efficiently (57). The oligomerization domain located in the C-terminal region of p53 also contributes to efficient Mdm2 binding and degradation (28, 35). Recently, the DBD of p53 has been reported to provide a secondary binding site for Mdm2 (49, 60). Several studies show that the central acidic domain of Mdm2 is involved in the interaction with p53 (30, 60, 68). The current model suggests that the N-terminal interaction between p53 and Mdm2 induces a conformational change in Mdm2 that promotes the binding of the acidic domain of Mdm2 to the DBD of p53. This second interaction between Mdm2 and p53 has also been shown to contribute to efficient ubiquitination (60).

The p53 gene is mutated in nearly 50% of all human cancers (23), resulting most commonly in single amino acid substitutions in the DBD (53; see also the International Agency for Research on Cancer TP53 mutation database). These mutant p53 proteins lose the ability to activate transcription, and they often become stable and so accumulate to high levels in tumor cells (7, 51). Since Mdm2 has been shown to retain the ability to bind and degrade mutant p53, the inability to transactivate the expression of Mdm2 has been proposed to underlie the stability of mutant p53 proteins (39). However, recent studies of mouse models expressing mutant p53 show that while this protein accumulates in tumors, it remains unstable in normal cells (31, 44). These results indicate that a failure to activate the expression of Mdm2 is not the underlying cause of mutant p53 stability in tumors. Moreover, high levels of Mdm2 are found in many tumor cell lines that express high levels of mutant p53 (45), and even though Mdm2 interacts with mutant p53, it still fails to degrade the protein in some tumor cell lines (6). Together, these observations suggest a more complex relationship between Mdm2 and p53 and indicate that the degradation of mutant p53 may be selectively compromised in tumor cells. Although they lose wild-type activity, there is compelling evidence that mutant p53 proteins acquire functions that enable them to contribute to malignant progression (31, 44, 51). It is therefore important to understand how the sta-

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▽ Published ahead of print on 1 October 2007.

bility of p53 mutant proteins is regulated and why they become stabilized in tumors.

Although previous studies have shown that tumor-derived p53 mutant forms can be targeted for degradation by Mdm2 when these proteins are overexpressed in cells (39), the recently identified contribution of the DBD of p53 to Mdm2 binding (49, 60) has suggested that mutant p53 proteins may show some alterations in their sensitivity to Mdm2. In this study, we have examined both Mdm2-dependent and Mdm2-independent pathways leading to the ubiquitination of mutant p53, and we show clear differences in the pathways that regulate mutant and wild-type p53 degradation.

MATERIALS AND METHODS

Cells and transfection. H1299 cells (p53-null human non-small-cell lung adenocarcinoma cells), MCF7-p53ΔII cells (human breast cancer cells expressing wild-type p53 and stably transfected with a construct encoding a p53 mutant protein lacking the conserved box II domain) (2), and $p53^{-/-}$ $mdm2^{-/-}$ double-knockout (DKO) mouse embryonic fibroblast cells (26) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, and antibiotics. Transfections of H1299 and DKO cells with plasmids were performed with Effectene transfection reagent (QIAGEN) or Lipofectamine 2000 (Invitrogen) by following the manufacturer's instructions. Cells were treated for the time periods indicated below with proteasome inhibitor MG132 (Sigma), Nutlin-3a (Cayman Chemical), or cycloheximide (Sigma) at the concentrations indicated below.

Cells were transfected with small interfering RNA (siRNA) oligonucleotides with Lipofectamine 2000 (Invitrogen). For the down-regulation of Cop1, ARF-BP1, and Mdm2 expression, cells were transfected twice with siRNA with 24 h between transfections and analyzed 48 h later. For the down-regulation of CHIP expression, cells were transfected twice with siRNA with 72 h between transfections and analyzed 72 h later. To assess degradation by Mdm2, cells were transfected twice with Mdm2-specific siRNA with HiPerfect reagent (QIAGEN) and analyzed 48 h later (see Fig. 6). For the down-regulation of Cop1, ARF-BP1, and CHIP expression, predesigned pools of four siRNA oligonucleotides (SMARTpool; Dharmacon) were used. In the control experiments, nontargeting siRNA was used (Dharmacon). Mdm2-specific siRNA oligonucleotides were described previously (55).

Plasmids. Plasmids expressing human wild-type p53 and p53 deletion mutant proteins (those lacking box I, II, III, IV, or V) (36), wild-type Mdm2 (11), Mdm2 with the C464A mutation (29), glutathione *S*-transferase (GST)–Mdm2 (16), Mdm2 with a deletion of the N-terminal p53 binding site (aa 58 to 89) (12), and Mdm2 lacking the RING finger domain (11) were described previously. Plasmids encoding Mdm2 with a deletion of the acidic domain (aa 212 to 296) and Mdm2 with zinc finger domain mutations C305A and C308A were generated by site-directed mutagenesis and verified by DNA sequencing. Plasmids encoding human p53 with the point mutation 273H or 175H and the 175H mutant p53 lacking box I were constructed by site-directed mutagenesis with the QuikChange kit (Stratagene, La Jolla, CA) and verified by DNA sequencing. The plasmid encoding hemagglutinin (HA)-tagged ubiquitin was kindly provided by R. Hay. pEGFP-N1 encoding green fluorescent protein (GFP) was obtained from Clontech (Palo Alto, CA).

Western blot analysis. Western blot analysis was carried out according to a method similar to one described previously (29). Human p53 was detected with the DO1 antibody (9), with the CM1 antibody (Novocastra), or with the 1801 antibody (Calbiochem). Human Mdm2 was detected with Ab-1 or Ab-2 antibodies (Calbiochem). Cop1-specific antibody was kindly provided by V. Dixit. ARF-BP1 antibody was kindly provided by M. Eilers. The CHIP-specific antibody was purchased from Abcam. Anti-GFP antibody and anti-HA antibody were purchased from Roche and Santa Cruz Biotechnology, respectively. Anti-Cdk4 antibody was purchased from Santa Cruz Biotechnology. Antiactin antibody and anti-Hsp90 antibody were purchased from Chemicon and Calbiochem, respectively.

Analysis of half-life of p53 by cycloheximide treatment. The day before transfection, 10^5 $p53^{-/-}$ $mdm2^{-/-}$ (DKO) cells were seeded into six-well plates. Cells were transfected with 150 ng of p53 with Effectene transfection reagent (QIAGEN). After 24 h, cells were treated with cycloheximide (50 μg/ml; Sigma) and collected at the time points indicated below. Cell lysates were subjected to Western blot analysis with p53-specific DO1 antibody.

Analysis of half-life of p53 by pulse-chase. The day before transfection, 10^5 $p53^{-/-}$ $mdm2^{-/-}$ (DKO) cells were seeded into six-well plates. Cells were transfected with 400 ng of p53 with Effectene transfection reagent (QIAGEN). The pulse-chase experiment was performed 24 h later. Cells were incubated in methionine/cysteine-free DMEM with 5% dialyzed serum (GIBCO) for 30 min. The medium was then removed and replaced with DMEM with [^{35}S]methionine-cysteine (50 μCi/ml; Promix [Amersham]) for 2 h. Cells were washed twice with phosphate-buffered saline (PBS) and chased with DMEM supplemented with 15 mg of methionine/liter and 24 mg of cysteine/liter (both from GIBCO) for the times indicated below. Cells were washed in PBS and lysed in NP-40 buffer. Cell lysates were immunoprecipitated with p53-specific DO1 antibody and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoprecipitation under native conditions. Immunoprecipitation was carried out according to a method similar to one described previously (29). Twenty-four hours before transfection, 3×10^5 H1299 cells were seeded into 6-cm plates. Cells were transfected with 0.6 μg of p53 and 1.2 μg of Mdm2 by using Lipofectamine 2000 reagent (Invitrogen). After 20 to 24 h, cells were treated with 10 μM MG132 for 4 h. Cells were washed in PBS and lysed in NP-40 buffer (0.5% NP-40, 120 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8) containing proteinase inhibitors (Complete; Roche). Immunoprecipitations were performed with Mdm2- or p53-specific antibodies bound to 30 μl of protein G-Sepharose for 1 or 2 h at 4°C. Mdm2 was immunoprecipitated with 0.3 to 0.6 μg of anti-Mdm2 antibodies Ab-2 (Calbiochem) and SMP-14 (Santa Cruz), and p53 was immunoprecipitated with 0.3 to 0.6 μg of 1801 and 421 antibodies (Calbiochem). Immunoprecipitated proteins were washed with NP-40 buffer and resuspended in 2× SDS sample buffer. Proteins from whole-cell extracts and immunoprecipitations were resolved by SDS-PAGE and analyzed by Western blotting with anti-p53 polyclonal antibody CM1 (Novocastra) and anti-Mdm2 Ab-1 and Ab-2 antibodies (Calbiochem).

In vivo ubiquitination of p53. DKO or H1299 cells were seeded at 60 to 80% confluence in 10-cm plates the day before transfection. Cells were transfected with 0.3 μg of p53 and 0.6 μg of HA-ubiquitin and with 0.15 μg of Mdm2 or pcDNA3 where indicated by using Effectene transfection reagent (QIAGEN). After 16 to 20 h, cells were treated with 20 to 30 μM MG132 for 5 h where indicated. Cells were washed twice in cold PBS and lysed in 200 μl of 1% SDS in Tris-buffered saline. After boiling and subjection to a vigorous vortex, extracts were supplemented with 400 μl of 1.5% Triton X-100 in Tris-buffered saline and incubated with 50 μl of protein G-Sepharose beads (Sigma) for 1 h at 4°C. Precleared samples were incubated with 50 μl of protein G-Sepharose beads preconjugated to p53-specific DO1 antibody for 2 h at 4°C. Then the beads were processed and associated proteins were analyzed using anti-HA antibody (Santa Cruz) and CM1 antibody (Novocastra).

In vitro ubiquitination of p53. The in vitro ubiquitination of p53 was carried out in a manner similar to that described previously (56). DHL5α *Escherichia coli* cells were transformed with pGEX-Mdm2 and grown at 37°C approximately to log phase. Protein expression was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside; 300 mM) for 3 h. Cells from 10 ml of an overnight culture were lysed in 5 ml of lysis buffer (50 mM Tris [pH 7.5], 100 mM NaCl, 1% Triton, and dithiothreitol (0.8 mg/ml) supplemented with phenylmethylsulfonyl fluoride) and sonicated. GST-Mdm2 was purified on 100 μl of glutathione-Sepharose beads (Amersham), mixed with 20 μl of p53 translated in vitro by using the TNT quick coupled transcription-translation system (Promega), and incubated at 4°C for 1 h. The beads were washed three times with 50 mM Tris, pH 7.5, and incubated with a mixture of 50 ng of mammalian E1 (Affiniti), 200 ng of human recombinant UbcH5B E2 (Affiniti), and 5 μg of ubiquitin or methylated ubiquitin (Sigma) in reaction buffer (50 mM Tris [pH 8], 2 mM dithiothreitol, 5 mM $MgCl_2$, 2 mM ATP). The reaction mixture was incubated at 37°C for 2 h, and then the reaction was stopped by the addition of 2× SDS sample buffer. Reaction products were resolved by SDS-PAGE and analyzed by Western blotting with anti-p53 DO1 antibody.

Immunofluorescence labeling. DKO or U2OS cells were seeded onto coverslips and transfected as described above for in vivo ubiquitination of p53. After 16 to 20 h, cells were treated with 20 μM MG132 for 5 h. Cells on coverslips were washed three times with PBS and then fixed in 4% paraformaldehyde for 10 min at room temperature. After fixation, cells were washed three times in PBS and permeabilized in PBS containing 0.2% Triton X-100 for 5 min. Cells were blocked in PBS containing 0.5% bovine serum albumin at room temperature for 30 min and then incubated for 2 h at room temperature with anti-p53 DO1 antibody in blocking solution. Cells were washed three times with PBS and incubated for 2 h at room temperature with fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody in blocking solution containing DAPI (4′,6′-diamidino-2-phenylindole 1 μg/ml; Sigma). Cells were washed three times with

PBS, and slides were mounted with Vectashield hard set (Vector Laboratories, Peterborough, United Kingdom).

Mdm2-mediated p53 degradation. The day before transfection, 10^5 DKO or H1299 cells were seeded into six-well plates. Cells were transfected with 150 ng of plasmid encoding wild-type p53 or the p53 mutant proteins indicated below and 450 ng (DKO cells) or 600 ng (H1299 cells) of plasmid encoding wild-type Mdm2 or the Mdm2 mutant proteins indicated below or the same amount of pcDNA3.1 (Invitrogen). To test whether Mdm2 lacking the RING finger domain could degrade p53 DBD mutant proteins lacking box I, 100 ng of the RING deletion form of Mdm2 was used in order to compensate for higher levels of expression of this mutant protein. Each transfection mixture also contained 50 ng of pEGFP-N1 to control for transfection efficiency. Cells were collected 30 to 40 h after transfection, washed with PBS, and lysed with 200 to 250 μl of 2× SDS sample buffer. Proteins were resolved by SDS-PAGE and analyzed by Western blotting with anti-p53 1801 antibody (Calbiochem), anti-Mdm2 Ab-1 and Ab-2 antibodies (Calbiochem), and anti-GFP antibody (Roche).

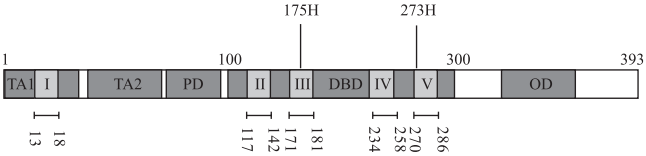

FIG. 1. Structural organization of the p53 protein. The locations of conserved boxes I to V and the corresponding deletions (ΔI to ΔV), the point mutations 175H and 273H, and the main domains of p53 are shown. TA1 and TA2, transactivation domains 1 and 2; PD, proline-rich domain; OD, oligomerization domain. Numbers indicate amino acid positions.

RESULTS

The mutant conformation of the DBD of p53 promotes the interaction of Mdm2 outside box I. Although the principal Mdm2 binding site is within the N terminus of p53, the DBD has recently also been shown to contribute to the interaction with Mdm2 (49, 60, 68). Most p53 mutations found in tumors occur in highly conserved regions of the DBD, boxes II, III, IV, and V (52). To assess the contribution of mutations affecting the DBD to the interaction with Mdm2, we utilized previously characterized p53 mutant proteins with deletions of the conserved boxes of the DBD (ΔI, ΔII, ΔIII, ΔIV, and ΔV) (36) (Fig. 1), as well as two tumor-derived mutant proteins with the hot-spot point mutation 175H or 273H. Similar to tumor-derived p53 mutant proteins (51), each of the p53 deletion mutant proteins fails to bind DNA and is therefore transcriptionally inactive (36). Many mutations alter the conformation of the DBD of p53 (5, 64), resulting in the exposure of an epitope recognized by the mutant-p53-specific antibody (pAb240), which does not recognize native wild-type p53 (67). Each of the deletion mutant proteins, like the 175H mutant form, adopts this mutant conformation associated with many tumor-derived p53 mutant proteins (37). The 273H mutant protein is recognized by both wild-type- and mutant-p53-specific antibodies and appears to display a flexible conformation (45, 61).

Previous studies showed that the p53 deletion mutant proteins retain the ability to interact with Mdm2 in vitro (36). To assess their ability to interact in vivo, we carried out a coimmunoprecipitation analysis of each of the deletion mutant forms of p53 and wild-type Mdm2 following coexpression in p53-null H1299 cells (Fig. 2A). Consistent with previous reports, wild-type Mdm2 associated with wild-type p53, whereas the deletion of box I almost completely disrupted this interaction (36). All of the p53 DBD deletion mutant proteins and wild-type p53 interacted similarly with Mdm2 (Fig. 2A), confirming that the change in conformation did not abolish the ability of Mdm2 to bind p53.

Mdm2 is known to interact with both the N terminus (12, 36) and the central DBD of p53 (49). To evaluate the contribution of the N-terminal binding site to the interaction between mutant p53 and Mdm2, we tested whether Mdm2 retained the ability to interact with p53 DBD mutant proteins lacking the conserved box I. The efficient association of Mdm2 with 175H and box V deletion mutant forms was similar to its association with wild-type p53 (Fig. 2B). Interestingly, although the deletion of the conserved box I almost completely abolished the interaction of wild-type p53 with Mdm2, conformational DBD 175H and box V deletion mutant proteins lacking the conserved box I clearly retained the ability to bind Mdm2, although the interaction was reduced compared to that of the DBD mutant proteins retaining box I (Fig. 2B). These results suggest that mutation in the DBD promotes an alternative interaction between Mdm2 and p53, which occurs outside box I of p53.

The RING domain of Mdm2 contributes to the interaction with DBD mutant forms of p53 lacking box I. Recent studies reported that Mdm2 can interact with the DBD of p53 via the acidic domain of Mdm2 (60, 68). To test whether the binding to p53 mutant proteins outside box I involves the acidic domain of Mdm2, we carried out a coimmunoprecipitation analysis with Mdm2 mutant proteins lacking this region. Similar to the results shown in Fig. 2B, wild-type Mdm2 readily associated with all of the p53 DBD mutant forms lacking box I but not with wild-type p53 lacking box I (Fig. 2C). However, the deletion of the acidic domain (aa 212 to 296) of Mdm2 did not prevent the interaction with wild-type p53 or the DBD mutant proteins lacking box I (Fig. 2C). These results therefore suggested that other regions of Mdm2 are important for interaction with mutant p53 (Fig. 2C).

To determine which region of Mdm2 interacts with mutant p53 outside box I, we examined several Mdm2 deletion mutations targeting functionally important regions: the N terminus, the zinc finger domain, and the RING finger domain (31). Mdm2 with the deletion of the N-terminal p53 binding site (aa 58 to 89) and Mdm2 with mutations of crucial amino acids in the zinc finger domain (C305A and C308A) interacted with the 175H mutant form of p53 lacking box I with an affinity similar to that of wild-type Mdm2 (Fig. 2D). However, the deletion of the C-terminal RING of Mdm2 abolished the ability of Mdm2 to interact with the 175H p53 mutant protein lacking box I (Fig. 2D), and this mutation also slightly decreased the interaction of wild-type p53 with Mdm2 (data not shown). Interestingly, the deletion of the C terminus of Mdm2 also significantly reduced the interaction with the 175H p53 mutant protein (which retains the N-terminal Mdm2 binding domain), suggesting that much of the interaction between Mdm2 and mutant p53 occurs via the RING finger domain (Fig. 2E). To examine whether the E3 activity of Mdm2 is required for the interaction with p53, we also used a mutant form of Mdm2 with a point mutation replacing cysteine residue 464 with alanine (C464A), previously shown to inhibit the ubiquitin ligase function of Mdm2 (16). The C464A protein retained the ability to

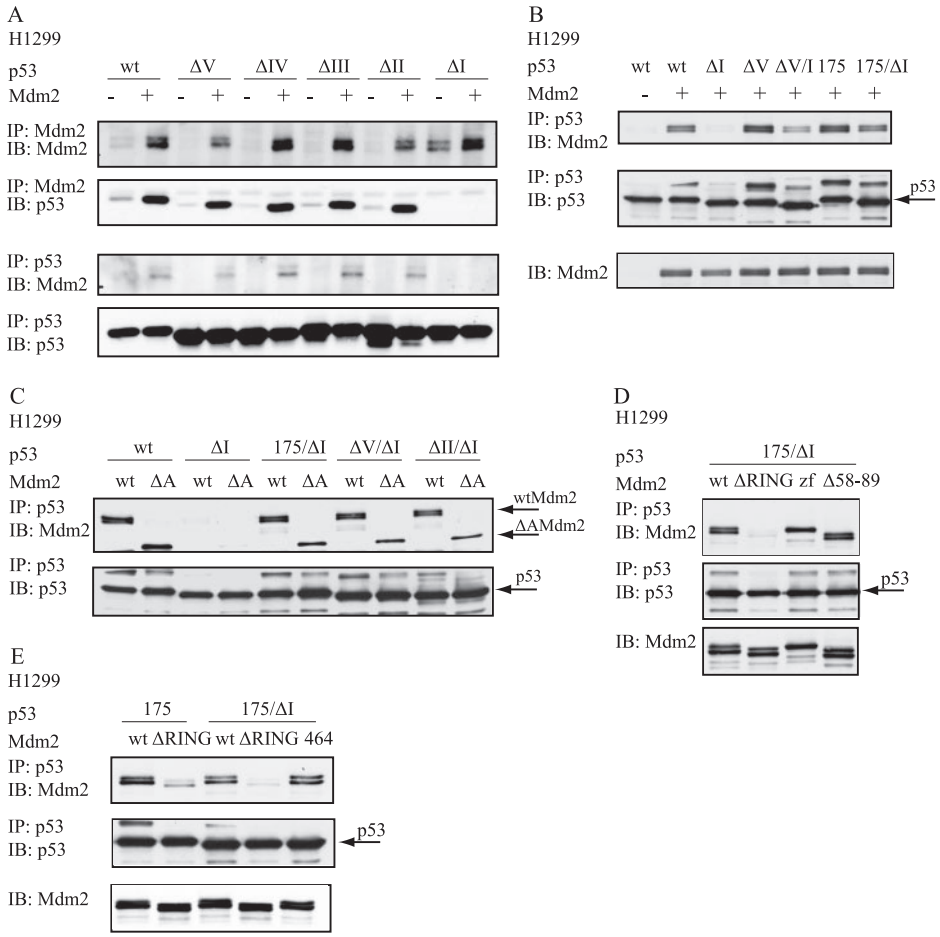

FIG. 2. The interaction of Mdm2 and p53 DBD mutant proteins occurs outside box I on p53 and involves the RING domain of Mdm2. (A) p53 mutant proteins with deletions of the conserved boxes of DBD interact with Mdm2. H1299 cells were transfected with plasmids encoding wild-type p53 (wt) or p53 deletion mutants and with an empty vector or Mdm2. Cell lysates were immunoprecipitated with anti-Mdm2 or anti-p53 antibodies and analyzed by immunoblotting (IB) with the indicated antibodies. IP, immunoprecitation; +, present; −, absent. (B) Mdm2 interacts with p53 DBD mutant proteins lacking conserved box I. H1299 cells were transfected with plasmids encoding wild-type p53 or the indicated mutants and with an empty vector or wild-type Mdm2. Samples immunoprecipitated with p53-specific antibodies and total cell lysates were analyzed with p53- and Mdm2-specific antibodies. 175, p53 carrying the 175H point mutation; 175/ΔI, p53 lacking box I and carrying the 175H point mutation; ΔV/I, p53 lacking boxes I and V. (C) The acidic domain of Mdm2 is not required for the interaction of Mdm2 with p53 DBD mutant proteins lacking box I. H1299 cells were transfected with wild-type p53 or the indicated p53 constructs along with wild-type Mdm2 (wtMdm2) or Mdm2 lacking the acidic domain (aa 212 to 296; ΔA or ΔAMdm2). The analysis of the interaction was carried out similarly to those described above. ΔII/I, p53 lacking boxes I and II. (D) The RING finger domain of Mdm2 is involved in the interaction of Mdm2 with the 175H p53 mutant protein outside box I. H1299 cells were transfected with the plasmids encoding p53 lacking box I and carrying the 175H point mutation and with wild-type Mdm2, Mdm2 lacking the RING finger domain (ΔRING), Mdm2 with mutations in the zinc finger domain (C305A and C308A; zf), or Mdm2 lacking the N-terminal p53 binding site (Δ58-89). The analysis of the interaction was carried out as described above. (E) The C464A Mdm2 mutant protein retains the ability to interact with the 175H p53 mutant form lacking box I. H1299 cells were transfected with the plasmids encoding 175H p53 mutant or the 175H p53 protein lacking box I and with wild-type Mdm2, Mdm2 lacking the RING finger domain (ΔRING), or Mdm2 with the mutation C464A (464). The analysis of the interaction was carried out similarly to those described above.

interact with the 175H mutant p53 lacking box I (Fig. 2E), indicating that the ubiquitin ligase activity of Mdm2 is not important but that the entire RING finger domain is required for the interaction of Mdm2 with mutant p53. Taken together, these results show that while both mutant and wild-type p53 proteins bind Mdm2, the mechanisms of binding are quite distinct.

p53 mutant proteins are less efficiently ubiquitinated by Mdm2 than wild-type p53 in vitro. To test whether the difference in the interaction between Mdm2 and mutant p53 affects the ability of Mdm2 to ubiquitinate mutant p53, we examined the efficiency of Mdm2-mediated ubiquitination of wild-type p53 and p53 mutant proteins in vitro. Interestingly, each of the p53 deletion mutant proteins was substantially less well ubiquitinated than wild-type p53 (Fig. 3A). The 175H p53 mutant protein exhibited a similar reduction in the ability to be ubiquitinated by Mdm2 (data not shown). This reduced ability of Mdm2 to ubiquitinate mutant p53 may reflect the differences in binding between the two proteins, since the interaction of wild-type p53 with the N terminus of Mdm2 has been suggested to be necessary to activate Mdm2 as a ubiquitin ligase (60).

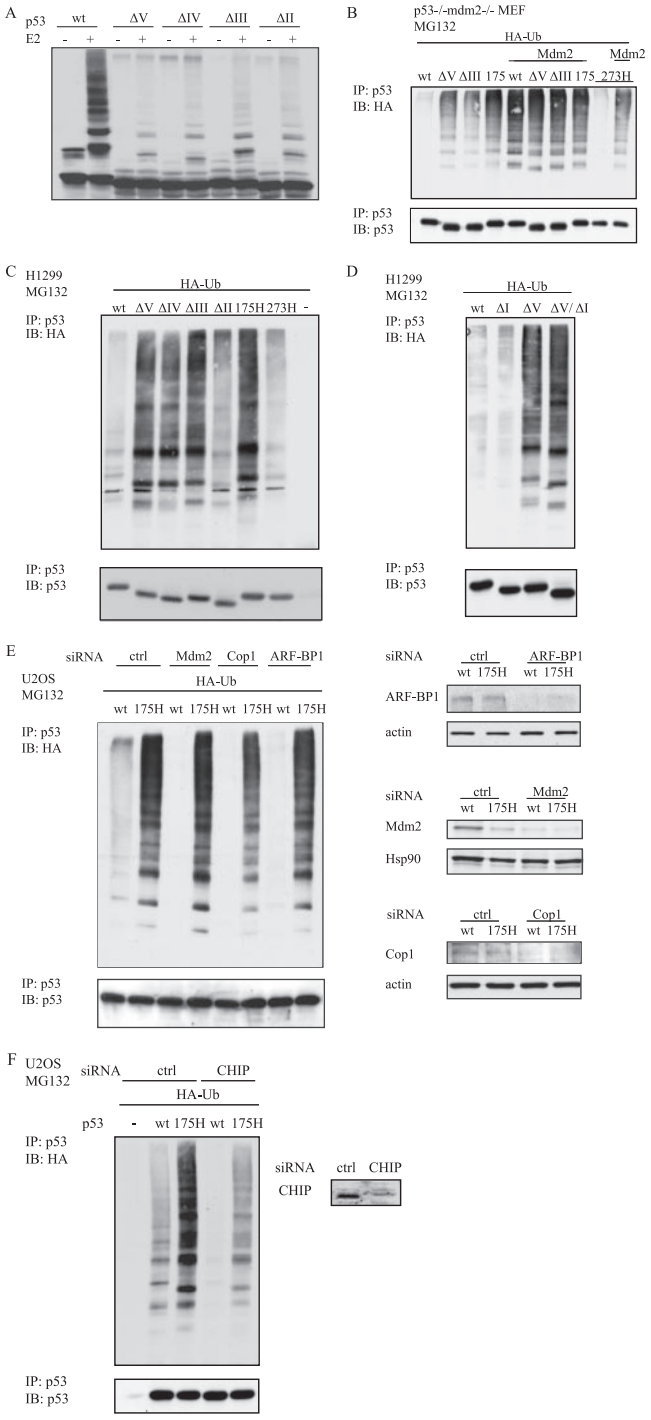

FIG. 3. Analysis of ubiquitination of p53 DBD mutant proteins. (A) p53 mutant proteins are less efficiently ubiquitinated by Mdm2 in vitro than wild-type p53. Wild-type p53 (wt) and p53 deletion mutant proteins (indicated by ΔII, ΔIII, ΔIV, and ΔV according to the box deleted) were in vitro-translated and bound to GST-Mdm2. In vitro ubiquitination reactions were carried out and analyzed by Western blotting with a p53-specific antibody (DO1). +, present; −, absent. (B) p53 mutant proteins are more highly ubiquitinated than wild-type p53 in the absence of Mdm2 in vivo. $p53^{-/-}$ $mdm2^{-/-}$ (DKO) cells were transfected with plasmids encoding wild-type p53 or the indicated p53 DBD mutants along with an empty vector or wild-type Mdm2 and with HA-ubiquitin (HA-Ub) and treated with MG132 for 5 h. Cells were lysed under denaturing conditions, immunoprecipitated with DO1 antibody, and subjected to Western blotting with either an anti-HA antibody or the p53-specific CM1 antibody. (C) p53 mutant proteins show enhanced ubiquitination in H1299 cells. H1299 cells were transfected with plasmids encoding wild-type p53 or the indicated p53 DBD mutants along with HA-ubiquitin and analyzed as described for panel B. (D) Box I is not required for the hyperubiquitination of the p53 protein lacking box V. H1299 cells were transfected with plasmids encoding wild-type p53 or the indicated p53 mutants along with HA-ubiquitin and analyzed as described for panel B. (E and F) Effect of siRNA-mediated reduction of Mdm2, Cop1, or ARF-BP1 (E) or CHIP (F) expression on the ubiquitination of wild-type p53 and the 175H p53 mutant protein. U2OS cells were transfected twice with nontargeting (control [ctrl]) or Mdm2-, Cop1-, and ARF-BP1-specific (E) and CHIP-specific (F) siRNA oligonucleotides with 24 h (E) or 72 h (F) between transfections. Cells were transfected with plasmids encoding wild-type p53 or the 175H p53 mutant along with HA-ubiquitin 24 h later, and the ubiquitination of p53 was analyzed as described for panel B. Direct Western blot analysis was carried out with the indicated antibodies. MEF, mouse embryonic fibroblasts; IP, immunoprecipitation; IB, immunoblotting.

p53 mutant proteins are more highly ubiquitinated in vivo independently of Mdm2 than wild-type p53. Our results so far showed that Mdm2 ubiquitinated mutant p53 less efficiently than wild-type p53 in vitro. To evaluate whether this decrease in efficiency leads to reduced ubiquitination of mutant p53 in cells, we carried out an in vivo ubiquitination assay with $p53^{-/-}$ $mdm2^{-/-}$ (DKO) mouse embryonic fibroblasts. While very little ubiquitination of wild-type p53 was detected in the absence of coexpressed Mdm2 (Fig. 3B) in these cells, all of the p53 mutant proteins that adopted an altered conformation (those with DBD deletions and 175H) were heavily ubiquitinated in the absence of Mdm2 (Fig. 3B). The expression of Mdm2 substantially increased the levels of ubiquitination of the wild-type p53 protein but had only a modest effect on the ubiquitination of the p53 mutant proteins (Fig. 3B), a result that is consistent with the less efficient ubiquitination of mutant proteins by Mdm2 seen in vitro (Fig. 3A). Interestingly, the p53 273H mutant protein that can retain a wild-type conformation was ubiquitinated much less than other p53 DBD mutant proteins in the absence of Mdm2 (Fig. 3B), implying that the sensitivity of p53 mutant proteins to Mdm2-independent ubiquitination depends on the conformation change in the DBD.

To confirm these observations with other deletion mutant proteins and different cells, we repeated the experiment in H1299 cells, which are p53 null and express low levels of endogenous Mdm2. Consistent with the results in DKO cells, conformational p53 mutant forms, but not wild-type p53, were highly ubiquitinated in the absence of additional Mdm2 (Fig. 3C). This hyperubiquitination was not reduced by the deletion of box I sequences in the p53 protein lacking box V (Fig. 3D). Interestingly, the deletion of box I did not reduce the residual ubiquitination of wild-type p53, suggesting that the wild-type protein is also sensitive to Mdm2-independent ubiquitination, although to a much lesser extent than mutant p53 (Fig. 3D).

Wild-type p53 is known to be ubiquitinated by many different E3 ligases (10, 13, 14, 32, 46, 54, 62, 66), some of which may also ubiquitinate mutant p53. We therefore examined the effect of the reduction of endogenous Mdm2, Cop1, ARF-BP1, or CHIP expression on the ubiquitination of wild-type p53 and the 175H mutant form of p53 in U2OS cells (Fig. 3E and F) and found similar results in H1299 cells (data not shown). As

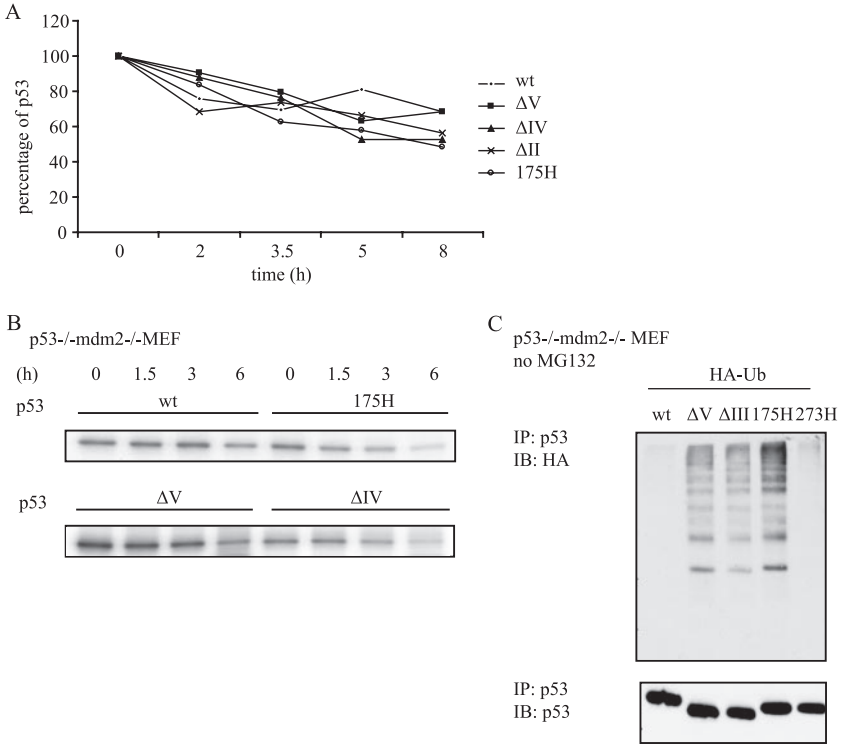

FIG. 4. The increase in the ubiquitination of p53 mutant proteins is not reflected in increased degradation. The half-lives of wild-type p53 and p53 DBD mutant proteins were analyzed. $p53^{-/-}$ $mdm2^{-/-}$ (DKO) cells were transfected with plamids encoding wild-type p53 (wtp53) or the indicated p53 DBD mutants. (A) Cells were treated with cycloheximide 24 h after transfection and collected at the indicated time points. Western blot analysis was carried out with the p53-specific DO1 antibody and an antiactin antibody. The results were quantitated using Scion image software (NIH). (B) Twenty-four hours after transfection, cells were pulsed with [^{35}S]methionine-cysteine and chased for the times indicated. Cell lysates were immunoprecipitated with the p53-specific antibody DO1 and analyzed by SDS-PAGE. MEF, mouse embryonic fibroblasts. (C) The ubiquitination of p53 DBD mutant proteins is detectable in cells in the absence of proteasome inhibitors. $p53^{-/-}$ $mdm2^{-/-}$ (DKO) cells were transfected with plasmids encoding wild-type p53 or the indicated p53 DBD mutants and with HA-ubiquitin (HA-Ub). Cells were lysed under denaturing conditions and immunoprecipitated with DO1 antibody. Western blot analysis was carried out with an anti-HA antibody and the p53-specific CM1 antibody. IP, immunoprecipitation; IB, immunoblotting.

expected, the down-regulation of each of the ubiquitin ligases reduced the ubiquitination of wild-type p53 to some extent. Consistent with the results shown in Fig. 3D, the reduction of Mdm2 did not affect mutant p53 ubiquitination (Fig. 3E), and similarly, ARF-BP1 appeared to contribute to the ubiquitination of only wild-type p53 (Fig. 3E). The down-regulation of Cop1 (Fig. 3E) and, most clearly, the chaperone-associated ubiquitin ligase CHIP (Fig. 3F) reduced the ubiquitination of mutant as well as wild-type p53. These results are in agreement with the findings in a previous report showing that CHIP can target both wild-type and mutant p53 for degradation (15) and were confirmed with another pair of CHIP-specific siRNA oligonucleotides and 175H and box V deletion mutant forms of p53 (data not shown).

The increase in ubiquitination of p53 mutant proteins is not reflected in increased degradation. Since the mutant p53 proteins were substantially ubiquitinated in DKO cells, we tested whether this increases their degradation rate by measuring the protein half-lives by two methods. First, we analyzed the half-lives of wild-type and mutant p53 by blocking protein synthesis with cycloheximide treatment (Fig. 4A). Wild-type p53 in normal cells has a very short half-life (around 0.5 h) (47), due to degradation by Mdm2 (22, 27). As expected, wild-type p53 had a prolonged half-life in Mdm2-null cells, whereas p53 proteins with deletions of conserved boxes and the 172H mutant p53 were very slightly less stable than wild-type p53. To confirm this observation, we carried out a pulse-chase analysis of the half-lives of wild-type p53, the p53 175H mutant protein, and the box V and box IV deletion mutant proteins in DKO cells (Fig. 4B). Again, all the p53 DBD mutant proteins were slightly less stable than wild-type p53 in this experiment, although the observed hyperubiquitination did not lead to the efficient degradation of mutant p53, as mutant p53 proteins were not fully degraded even after 6 h (Fig. 4B). The lack of correlation between the ubiquitination of mutant p53 and the degradation rate suggests that ubiquitinated mutant p53 should be detected in cells even in the absence of proteasome inhibitors to block degradation. This prediction was confirmed in DKO cells, in which ubiquitinated forms of conformationally mutated p53 proteins, but not wild-type p53, accumulated even in the absence of proteasomal inhibition (Fig. 4C).

The ubiquitination of the C terminus of p53 has been shown to contribute to the exposure of the nuclear export signal and to result in the nuclear export of p53 (8, 33). We therefore tested whether the Mdm2-independent ubiquitination of p53 mutant proteins affects their localization. In DKO cells, wild-type p53 and the p53 273H mutant protein, which are not ubiquitinated under these conditions (Fig. 3B), were located in

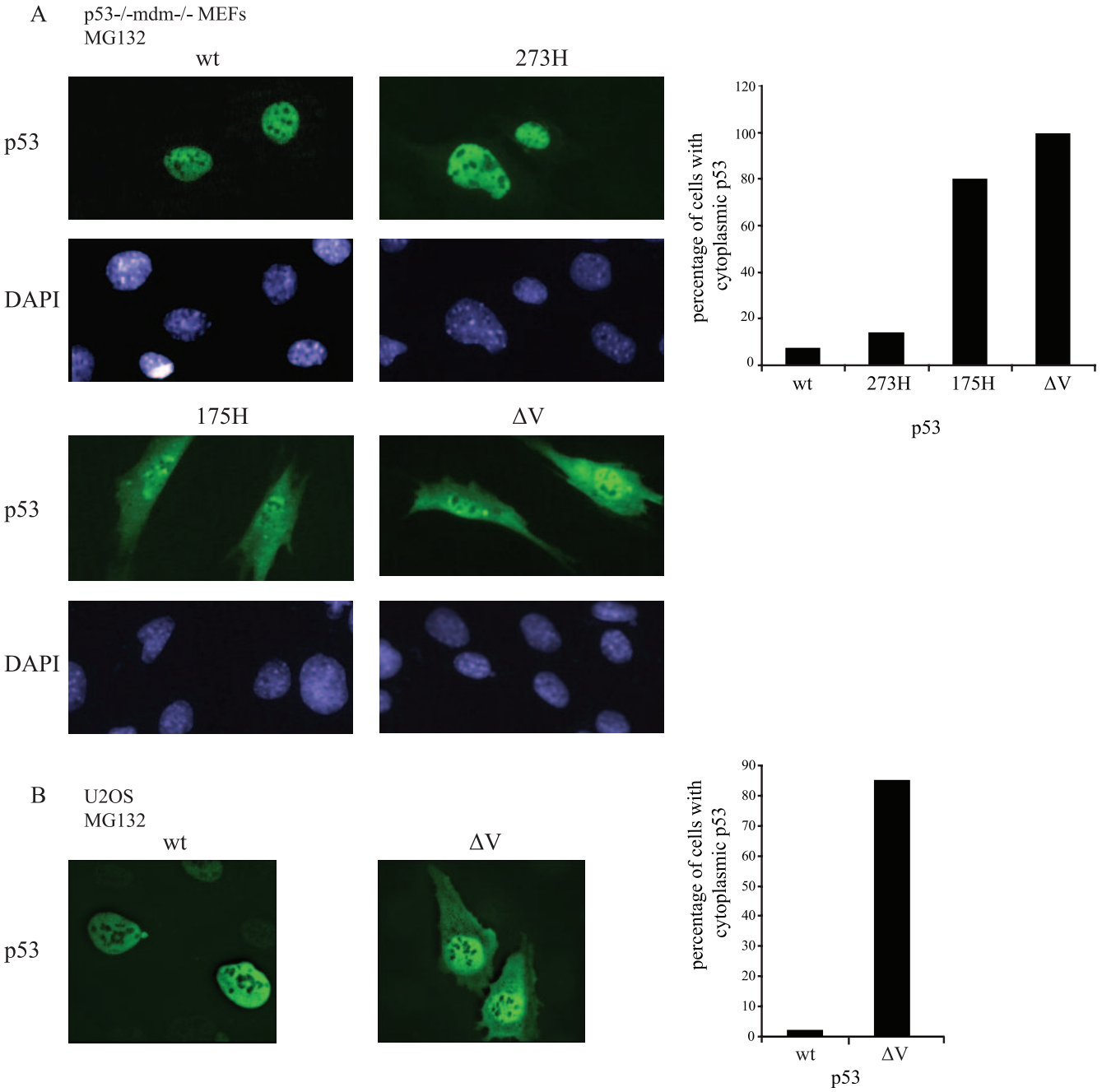

FIG. 5. Ubiquitinated p53 DBD mutant proteins localize to the cytoplasm. $p53^{-/-}$ $mdm2^{-/-}$ (DKO) cells (A) or U2OS cells (B) were transfected with plasmids encoding wild-type p53 (wt) or the indicated p53 DBD mutants along with HA-ubiquitin. Cells were treated with MG132 for 5 h and stained with anti-p53 DO1 and DAPI. The bar graph shows average percentages of cells with cytoplasmic p53. MEFs, mouse embryonic fibroblasts.

the nucleus (Fig. 5A). However, the ubiquitinated 175H and box V deletion mutant proteins were present in the cytoplasm in the majority of cells (Fig. 5A). This observation was confirmed in U2OS cells (Fig. 5B). These results show that although the Mdm2-independent ubiquitination of p53 mutant proteins does not target them for degradation, it is associated with cytoplasmic accumulation. While this effect may reflect enhanced nuclear export, the possibility that mutant p53 is specifically ubiquitinated in the cytoplasm cannot be excluded. Similarly, a recent report has also shown that the ubiquitination of cancer-derived p53 mutant proteins is associated with enhanced cytoplasmic localization (42).

p53 DBD mutant forms are degraded by Mdm2 in vivo independently of the N-terminal interaction. Our results suggest that p53 mutant proteins are poorly ubiquitinated by Mdm2 but can be efficiently ubiquitinated in vivo by another E3 ligase. However, this ubiquitination does not result in the rapid degradation of the mutant p53 proteins, possibly because

they are not targeted to the proteasome. Despite the weak effect of Mdm2 on the ubiquitination of the mutant p53 proteins, previous reports have shown clearly that Mdm2 can contribute to the degradation of the p53 DBD mutant forms in cells (27, 39). Taken together, these data suggest that Mdm2 may play a role in the degradation of mutant p53 proteins that is distinct from ubiquitination. Indeed, two roles for Mdm2 in the degradation of wild-type p53 have been suggested recently, one to ubiquitinate p53 and one to deliver it to the proteasomes (4, 30, 69). We therefore sought to test whether Mdm2 could target p53 mutant proteins that have been ubiquitinated by another E3 ligase to the proteasomes, an activity that may be independent of the ubiquitin ligase function of Mdm2.

As discussed above, the N-terminal Mdm2 binding region is not required for the ubiquitination of p53 mutant proteins by the Mdm2-independent mechanism. Therefore, we tested the ability of Mdm2 to degrade p53 proteins lacking box I in DKO cells. Wild-type p53 is efficiently targeted to the proteasomes by Mdm2, whereas the deletion of box I rendered p53 resistant to degradation. This result was consistent with the crucial role of the N-terminal interaction between p53 and Mdm2 in the ability of Mdm2 to ubiquitinate and degrade wild-type p53 (Fig. 6A). Consistent with the findings in a previous report (28), all p53 DBD mutant forms tested (the box II and box V deletion mutant proteins and the 175H mutant protein) were efficiently degraded by Mdm2, similarly to wild-type p53 (Fig. 6A). Interestingly, Mdm2 was also able to target p53 DBD mutant proteins lacking box I for degradation (although clearly to a lesser extent than p53 DBD mutant proteins that retained box I) (Fig. 6A), suggesting that Mdm2 can directly target p53 mutants that have been ubiquitinated by other E3 ligases to the proteasomes. This mechanism of degradation does not require the N-terminal interaction between mutant p53 and Mdm2, suggesting that the E3 activity of Mdm2 does not need to be activated.

To support these observations, we examined the effects of some Mdm2 mutations. The N terminus of p53 has been reported to interact only with the N-terminal p53 binding site on Mdm2 (40, 68). Therefore, if box I is not required for the degradation of p53 mutant proteins by Mdm2, Mdm2 with a deletion of the N-terminal p53 binding site should still be able to degrade mutant p53. To test this, we expressed wild-type p53 or the box V or 175H mutant protein along with wild-type Mdm2 or Mdm2 lacking the N-terminal p53 binding site (aa 58 to 89). Consistent with previous reports, the N-terminal p53 binding site in Mdm2 was required for the efficient degradation of wild-type p53 (Fig. 6B). However, in agreement with the results of the experiment described above, the Mdm2 mutant protein lacking the binding site retained some ability to degrade the p53 mutant proteins (Fig. 6B). These results are consistent with a model in which mutant p53 that has been ubiquitinated independently of Mdm2 can be targeted to the proteasome by Mdm2 and in which this activity of Mdm2 does not require the interaction between the N-terminal domains of Mdm2 and p53. However, we consistently noted that this degradation of mutant p53 was less efficient than that seen when the N-terminal interaction sites were intact, suggesting that the mechanisms that degrade wild-type p53 can still contribute to the degradation of the mutant p53 proteins. This dependence on N-terminal binding was seen much more strongly in H1299 cells, in which the deletion of box I sequences effectively prevented Mdm2-mediated degradation of the p53 DBD mutant proteins with 175H and the box V deletion (Fig. 6C). It would therefore seem possible that the efficiency with which Mdm2 can target ubiquitinated p53 for degradation in the absence of the N-terminal binding varies among cell types.

Our binding studies (Fig. 2) showed that the interaction of mutant p53 that is independent of the N-terminal binding region requires the C-terminal RING domain. We therefore tested the ability of Mdm2 lacking this region to target the degradation of the DBD-mutated p53 proteins with 175H and the box V deletion in DKO cells. As expected, the deletion of the N-terminal Mdm2 binding region in wild-type p53 prevented degradation by all forms of Mdm2 (Fig. 6D). As shown above, wild-type Mdm2 retained some ability to degrade p53 DBD mutant forms lacking the N-terminal Mdm2 binding region. This activity was lost by the deletion of the RING domain of Mdm2 but was retained by the Mdm2 protein with the C464A mutation, which has lost E3 activity but still binds to the DBD mutant proteins (Fig. 2E). These results therefore support the model in which Mdm2 can target the degradation of the mutant p53 proteins through a mechanism that requires a RING domain interaction but not E3 activity of Mdm2.

Most of the studies described so far depended on the transient expression of p53 and Mdm2 mutant proteins. In order to test the contribution of the N-terminal interaction of p53 and Mdm2 to the degradation of wild-type p53 and mutant p53 stably expressed in cells, we turned to MCF7-p53ΔII cells, which express endogenous wild-type p53 and stably express exogenous p53 lacking box II (2). These cells express endogenous Mdm2 to a level that can drive the degradation of both the wild-type and mutant p53, although somewhat higher basal levels of mutant p53 are maintained in these cells, and the exposure of these cells to stress leads to the coordinate stabilization of both the mutant and wild-type p53 proteins (2). In this system, p53 lacking the N-terminal Mdm2 binding domain (box I) is expressed at high levels and not further stabilized in response to stress (2).

To examine the contribution of Mdm2 to the stability of wild-type p53 and the box II mutant p53 in this system, we took an siRNA-mediated approach to reduce endogenous Mdm2 levels. Consistent with the ability of Mdm2 to degrade both wild-type and mutant p53, wild-type p53 and the p53 box II mutant form were stabilized to similar extents when Mdm2 expression was down-regulated (Fig. 6E). To examine the effect of disrupting the N-terminal p53-Mdm2 interaction, we used Nutlin-3, a small-molecule inhibitor that specifically binds to the p53 binding pocket in the N terminus of Mdm2, preventing the interaction through the N-terminal domains and stabilizing p53 (57). As expected, the wild-type p53 protein was substantially stabilized upon treatment with Nutlin-3, consistent with a major role of the N-terminal interaction between p53 and Mdm2 in the regulation of wild-type p53 stability (Fig. 6F). Interestingly, the p53 mutant protein lacking box II was stabilized by Nutlin-3 to a much lesser extent than wild-type p53 (Fig. 6F, short exposure). This finding is consistent with the observation that Mdm2 can degrade p53 mutant proteins through a mechanism that does not depend on the N-terminal interaction (Fig. 6A and B). The fact that wild-type p53 is stabilized more by treatment with Nutlin-3 than mutant p53

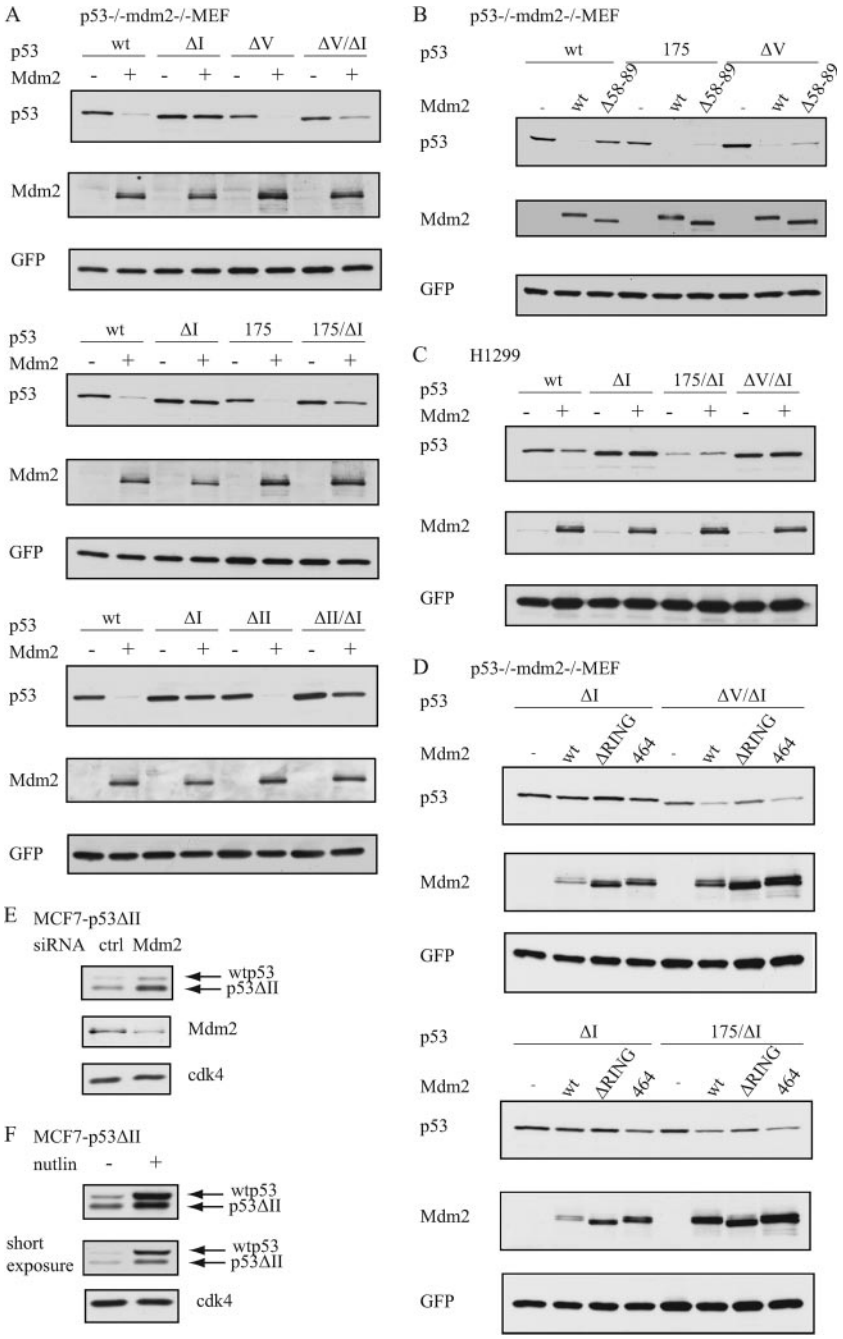

FIG. 6. The degradation of p53 DBD mutant proteins by Mdm2 in cells is independent of the N-terminal interaction. $p53^{-/-}$ $mdm2^{-/-}$ (DKO) cells (A, B, and D) or H1299 cells (C) were transfected with the indicated plasmids. Cells were cotransfected with GFP to control for transfection efficiency. Western blot analysis was carried out with p53-specific (1801), Mdm2-specific (Ab-1 and Ab-2), and GFP-specific antibodies. (A) p53 DBD mutant proteins lacking box I are degraded by Mdm2. DKO cells were transfected with plasmids encoding wild-type p53 (wt) or the indicated p53 DBD mutants along with an empty vector or wild-type Mdm2. +, present; −, absent. MEF, mouse embryonic fibroblasts. (B) Mdm2 lacking the N-terminal p53 binding site retains the ability to degrade p53 DBD mutants. DKO cells were transfected with plasmids encoding wild-type p53 or the indicated p53 DBD mutants along with an empty vector, wild-type Mdm2, or Mdm2 lacking the N-terminal p53 binding site (Δ58–89). (C) The deletion of box I prevents the Mdm2-mediated degradation of p53 DBD mutant proteins in H1299 cells. H1299 cells were transfected with plasmids encoding wild-type p53 or the indicated p53 mutants along with an empty vector or wild-type Mdm2. (D) Mdm2 lacking the RING finger domain does not degrade p53 DBD mutant proteins lacking box I. DKO cells were transfected with plasmids encoding p53 lacking box I and the 175H p53 mutant lacking box I along with an empty vector, wild-type Mdm2, Mdm2 lacking the RING finger domain (ΔRING), or the Mdm2 C464A mutant (464). (E) The down-regulation of endogenous Mdm2 stabilizes wild-type p53 and p53 lacking box II (p53ΔII) similarly. MCF7-p53ΔII cells, which have been stably transfected with the p53 mutant lacking box II and express endogenous wild-type p53, were transfected twice with nontargeting (control [ctrl]) or Mdm2-specific siRNA oligonucleotides. Cells were lysed 48 h later, and Western blot analysis was carried out with p53-specific (1801), Mdm2-specific, or cdk4-specific antibodies. (F) p53 lacking box II is less stabilized by Nutlin-3 treatment than wild-type p53. MCF7-ΔII cells were treated with 20 μM Nutlin-3 for 6.5 h. Cell lysates were analyzed for p53 levels by Western blotting with 1801 antibody.

may have an important implication for cancer treatments of tumors expressing mutant and wild-type p53.

DISCUSSION

Many of the p53 mutations found in tumors affect the DBD of p53, which partially or completely distorts its conformation (5, 64). Mutant p53 often accumulates to high levels in tumor cells (51), although the reason why mutant p53 is not degraded in cancer cells remains unclear. While these mutant proteins do not activate the expression of Mdm2, it is apparent that normal cells retain sufficient Mdm2 levels to keep mutant p53 levels low. In mice engineered to express only mutant p53, the stabilization of p53 is seen only in tumors (31, 44). It is possible that the stabilization of mutant p53 in tumors is related to the expression of ARF, an inhibitor of Mdm2 that is specifically activated in tumors (34). However, siRNA-mediated inhibition of ARF expression in a tumor cell line expressing high levels of mutant p53 did not decrease the stability of the p53 (H. Horn and K. H. Vousden, unpublished data), suggesting that ARF is not the only determinant of the stability of mutant p53. The results described here indicate that mutant and wild-type p53 can be degraded through overlapping, but distinct, pathways. It is therefore possible that the selective stabilization of mutant p53 reflects a tumor-specific defect in the pathways that target mutant p53 for degradation. However, it is clear that other factors contribute to the stability of mutant p53 proteins in cancers, since p53 forms encoded by a gene mutated at codon 273, which were ubiquitinated like wild-type p53 in this study, are frequently found to be stabilized in human cancers.

We found that while Mdm2 retains the ability to interact with conformationally altered p53 DBD mutant proteins, this interaction appeared to be shifted from the N-terminal binding sites that are used predominantly by wild-type p53 to an alternative site on mutant p53. Several recent studies have shown that p53 can bind to Mdm2 through the DBD (49, 60, 68), and it seems likely that the conformational shift in mutant p53 reveals this binding site, thereby enhancing the interaction of mutant p53 with Mdm2 in a conserved box I-independent manner.

Mdm2 also contains several sites of interaction with p53 (12, 30, 60, 68). Recent studies have suggested that N-terminal interaction between Mdm2 and p53 triggers a conformational switch in Mdm2 that is required to promote a second interaction that involves the acidic domain of Mdm2 and the DBD of p53 (60). Taken together with the results of our studies, these findings appear to demonstrate that a conformational change in both p53 and Mdm2 contributes to their interaction through regions distinct from the N termini.

While other studies have shown the importance of the acidic domain of Mdm2 in alternative interactions with p53 (30, 60, 68), we found that the RING finger domain of Mdm2 is involved in binding to mutant p53. Although the RING finger domain of Mdm2 is required for ubiquitin ligase activity (16), this function is not necessary for the binding to mutant p53, as demonstrated by the E3-inactive form of Mdm2 with the point mutation C464A. Furthermore, the interaction of Mdm2 with mutant p53 does not lead to the strong ubiquitination of p53 that is seen following interaction with the wild-type p53 protein. This may reflect, to some extent, a lack of interaction with the N terminus of Mdm2, which has been suggested to be required to activate E3 function (60).

Interestingly, we found that despite the reduced sensitivity to Mdm2-mediated ubiquitination, mutant p53 acquired sensitivity to another E3 ligase that selectively recognizes and ubiquitinates conformationally altered mutant p53 proteins. The increased ubiquitination of some p53 mutant proteins with altered conformations has been noted previously (42, 50), although we have shown here that this effect is not Mdm2 dependent. Our data imply that Mdm2-independent ubiquitination does not result in the efficient degradation of mutant p53 proteins, although in agreement with the results in a recent report (42), we have shown that it is associated with enhanced cytoplasmic localization. A number of other E3 ligases that can target wild-type p53 have been described recently (10, 13, 14, 32, 46, 54, 62, 66), and it is possible that some of them are responsible for the ubiquitination of mutant p53. Conformationally mutated p53 proteins in cells are specifically recognized by molecular chaperones Hsp90 and Hsc70 (17, 63), which can present substrates to the ubiquitin ligase CHIP (25). Previous studies have shown that both wild-type and mutant p53 proteins are targeted to the proteasomes by CHIP (15), and our study shows that the down-regulation of CHIP expression reduces the ubiquitination of both wild-type and mutant p53. These results suggest that CHIP may play a role in the ubiquitination and degradation of mutant p53 and that the reduced expression of CHIP in some cancers (see the Oncomine database) may contribute to the enhanced stability of mutant p53 proteins. However, the difference in the ubiquitination of mutant and wild-type p53 proteins suggests that there may be E3 ligases that target only mutant p53 or that wild-type and mutant p53 proteins differ in their sensitivity to deubiquitinases.

Our results show that although the ability of Mdm2 to ubiquitinate p53 mutant proteins is compromised, Mdm2 can still efficiently degrade mutant forms of p53. Interestingly, the ability of Mdm2 to degrade did not absolutely require N-terminal interaction between mutant p53 and Mdm2 and appears to be independent of the ability of Mdm2 to function as an E3 ligase. Previous reports have also suggested that the ubiquitination function of Mdm2 can be uncoupled from its ability to target for degradation, as some Mdm2 mutant forms can ubiquitinate p53 but cannot degrade it (30, 69). Our study provides further evidence that Mdm2 can play a postubiquitination role in degrading p53. The details of such a function of Mdm2 are not yet clear but have been suggested to involve cooperation with hHR23A, a protein thought to be an adaptor between ubiquitinated substrates and the proteasome (4, 19). Another study has also shown that Mdm2 can play a role in transport to the proteasomes by interacting directly with the C8 subunit of the proteasomes (48).

Our data suggest that the regulation of the stability of mutant p53 differs from that of wild-type p53. Identifying the E3 ligase responsible for the hyperubiquitination of mutant p53 and investigating the mechanism of Mdm2-mediated delivery to the proteasomes may have important implications for identifying mechanisms to specifically down-regulate mutant, but not wild-type, p53 in tumors. This specific down-regulation may have therapeutic advantages, since mutant p53 proteins expressed in cancer cells show a clear ability to

promote various aspects of tumorigenesis, including metastatic spread (31, 44).

ACKNOWLEDGMENTS

We are grateful to Arnie Levine, Stjepan Uldrijan, and Mary Hanlon for Mdm2 constructs and to Robert Ludwig for help with experiments. We thank Ron Hay for the HA-tagged ubiquitin constructs, Vishva Dixit for Cop1-specific antibody, Martin Eilers for ARF-BP1-specific antibody, and all the members of the Tumor Suppressor Laboratory for their help and advice during the course of this study.

We also thank Cancer Research UK and acknowledge FP6 grant "Active p53" for funding support.

REFERENCES

1. **Alarcon-Vargas, D., and Z. Ronai.** 2002. p53-Mdm2: the affair that never ends. Carcinogenesis **23:**541–547.
2. **Ashcroft, M., M. H. Kubbutat, and K. H. Vousden.** 1999. Regulation of p53 function and stability by phosphorylation. Mol. Cell. Biol. **19:**1751–1758.
3. **Boyd, S. D., K. Y. Tsai, and T. Jacks.** 2000. An intact HDM2 RING-finger domain is required for nuclear exclusion of p53. Nat. Cell Biol. **2:**563–568.
4. **Brignone, C., K. E. Bradley, A. F. Kisselev, and S. R. Grossman.** 2004. A post-ubiquitination role for MDM2 and hHR23A in the p53 degradation pathway. Oncogene **23:**4121–4129.
5. **Bullock, A. N., and A. R. Fersht.** 2001. Rescuing the function of mutant p53. Nat. Rev. Cancer **1:**68–76.
6. **Buschmann, T., T. Minamoto, N. Wagle, S. Y. Fuchs, V. Adler, M. Mai, and Z. Ronai.** 2000. Analysis of JNK, Mdm2 and p14(ARF) contribution to the regulation of mutant p53 stability. J. Mol. Biol. **295:**1009–1021.
7. **Cadwell, C., and G. P. Zambetti.** 2001. The effects of wild-type p53 tumor suppressor activity and mutant p53 gain-of-function on cell growth. Gene **277:**15–30.
8. **Carter, S., O. Bischof, A. Dejean, and K. H. Vousden.** 2007. C-terminal modifications regulate MDM2 dissociation and nuclear export of p53. Nat. Cell Biol. **9:**428–435.
9. **Ceskova, P., H. Chichger, M. Wallace, B. Vojtesek, and T. R. Hupp.** 2006. On the mechanism of sequence-specific DNA-dependent acetylation of p53: the acetylation motif is exposed upon DNA binding. J. Mol. Biol. **357:**442–456.
10. **Chen, D., N. Kon, M. Li, W. Zhang, J. Qin, and W. Gu.** 2005. ARF-BP1/Mule is a critical mediator of the ARF tumor suppressor. Cell **121:**1071–1083.
11. **Chen, J., J. Lin, and A. J. Levine.** 1995. Regulation of transcription functions of the p53 tumor suppressor by the mdm-2 oncogene. Mol. Med. **1:**142–152.
12. **Chen, J., V. Marechal, and A. J. Levine.** 1993. Mapping of the p53 and mdm-2 interaction domains. Mol. Cell. Biol. **13:**4107–4114.
13. **Corcoran, C. A., Y. Huang, and M. S. Sheikh.** 2004. The p53 paddy wagon: COP1, Pirh2 and MDM2 are found resisting apoptosis and growth arrest. Cancer Biol. Ther. **3:**721–725.
14. **Dornan, D., I. Wertz, H. Shimizu, D. Arnott, G. D. Frantz, P. Dowd, K. O'Rourke, H. Koeppen, and V. M. Dixit.** 2004. The ubiquitin ligase COP1 is a critical negative regulator of p53. Nature **429:**86–92.
15. **Esser, C., M. Scheffner, and J. Hohfeld.** 2005. The chaperone-associated ubiquitin ligase CHIP is able to target p53 for proteasomal degradation. J. Biol. Chem. **280:**27443–27448.
16. **Fang, S., J. P. Jensen, R. L. Ludwig, K. H. Vousden, and A. M. Weissman.** 2000. Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. J. Biol. Chem. **275:**8945–8951.
17. **Finlay, C. A., P. W. Hinds, T. H. Tan, D. Eliyahu, M. Oren, and A. J. Levine.** 1988. Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. Mol. Cell. Biol. **8:**531–539.
18. **Geyer, R. K., Z. K. Yu, and C. G. Maki.** 2000. The MDM2 RING-finger domain is required to promote p53 nuclear export. Nat. Cell Biol. **2:**569–573.
19. **Glockzin, S., F. X. Ogi, A. Hengstermann, M. Scheffner, and C. Blattner.** 2003. Involvement of the DNA repair protein hHR23 in p53 degradation. Mol. Cell. Biol. **23:**8960–8969.
20. **Gu, J., L. Nie, D. Wiederschain, and Z. M. Yuan.** 2001. Identification of p53 sequence elements that are required for MDM2-mediated nuclear export. Mol. Cell. Biol. **21:**8533–8546.
21. **Harms, K. L., and X. Chen.** 2006. The functional domains in p53 family proteins exhibit both common and distinct properties. Cell Death Differ. **13:**890–897.
22. **Haupt, Y., R. Maya, A. Kazaz, and M. Oren.** 1997. Mdm2 promotes the rapid degradation of p53. Nature **387:**296–299.
23. **Hollstein, M., D. Sidransky, B. Vogelstein, and C. C. Harris.** 1991. p53 mutations in human cancers. Science **253:**49–53.
24. **Honda, R., H. Tanaka, and H. Yasuda.** 1997. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett. **420:**25–27.
25. **Jiang, J., C. A. Ballinger, Y. Wu, Q. Dai, D. M. Cyr, J. Hohfeld, and C. Patterson.** 2001. CHIP is a U-box-dependent E3 ubiquitin ligase: identification of Hsc70 as a target for ubiquitylation. J. Biol. Chem. **276:**42938–42944.
26. **Jones, S. N., A. E. Roe, L. A. Donehower, and A. Bradley.** 1995. Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature **378:**206–208.
27. **Kubbutat, M. H., S. N. Jones, and K. H. Vousden.** 1997. Regulation of p53 stability by Mdm2. Nature **387:**299–303.
28. **Kubbutat, M. H., R. L. Ludwig, M. Ashcroft, and K. H. Vousden.** 1998. Regulation of Mdm2-directed degradation by the C terminus of p53. Mol. Cell. Biol. **18:**5690–5698.
29. **Kubbutat, M. H., R. L. Ludwig, A. J. Levine, and K. H. Vousden.** 1999. Analysis of the degradation function of Mdm2. Cell Growth Differ. **10:**87–92.
30. **Kulikov, R., M. Winter, and C. Blattner.** 2006. Binding of p53 to the central domain of Mdm2 is regulated by phosphorylation. J. Biol. Chem. **281:**28575–28583.
31. **Lang, G. A., T. Iwakuma, Y. A. Suh, G. Liu, V. A. Rao, J. M. Parant, Y. A. Valentin-Vega, T. Terzian, L. C. Caldwell, L. C. Strong, A. K. El-Naggar, and G. Lozano.** 2004. Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. Cell **119:**861–872.
32. **Leng, R. P., Y. Lin, W. Ma, H. Wu, B. Lemmers, S. Chung, J. M. Parant, G. Lozano, R. Hakem, and S. Benchimol.** 2003. Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation. Cell **112:**779–791.
33. **Lohrum, M. A., D. B. Woods, R. L. Ludwig, E. Balint, and K. H. Vousden.** 2001. C-terminal ubiquitination of p53 contributes to nuclear export. Mol. Cell. Biol. **21:**8521–8532.
34. **Lowe, S. W., and C. J. Sherr.** 2003. Tumor suppression by Ink4a-Arf: progress and puzzles. Curr. Opin. Genet. Dev. **13:**77–83.
35. **Maki, C. G.** 1999. Oligomerization is required for p53 to be efficiently ubiquitinated by MDM2. J. Biol. Chem. **274:**16531–16535.
36. **Marston, N. J., T. Crook, and K. H. Vousden.** 1994. Interaction of p53 with MDM2 is independent of E6 and does not mediate wild type transformation suppressor function. Oncogene **9:**2707–2716.
37. **Marston, N. J., J. R. Jenkins, and K. H. Vousden.** 1995. Oligomerisation of full length p53 contributes to the interaction with mdm2 but not HPV E6. Oncogene **10:**1709–1715.
38. **Michael, D., and M. Oren.** 2003. The p53-Mdm2 module and the ubiquitin system. Semin. Cancer Biol. **13:**49–58.
39. **Midgley, C. A., and D. P. Lane.** 1997. p53 protein stability in tumour cells is not determined by mutation but is dependent on Mdm2 binding. Oncogene **15:**1179–1189.
40. **Moll, U. M., and O. Petrenko.** 2003. The MDM2-p53 interaction. Mol. Cancer Res. **1:**1001–1008.
41. **Momand, J., H. H. Wu, and G. Dasgupta.** 2000. MDM2: master regulator of the p53 tumor suppressor protein. Gene **242:**15–29.
42. **Nie, L., M. Sasaki, and C. G. Maki.** 2007. Regulation of p53 nuclear export through sequential changes in conformation and ubiquitination. J. Biol. Chem. **282:**14616–14625.
43. **Oliner, J. D., J. A. Pietenpol, S. Thiagalingam, J. Gyuris, K. W. Kinzler, and B. Vogelstein.** 1993. Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. Nature **362:**857–860.
44. **Olive, K. P., D. A. Tuveson, Z. C. Ruhe, B. Yin, N. A. Willis, R. T. Bronson, D. Crowley, and T. Jacks.** 2004. Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. Cell **119:**847–860.
45. **Peng, Y., L. Chen, C. Li, W. Lu, and J. Chen.** 2001. Inhibition of MDM2 by hsp90 contributes to mutant p53 stabilization. J. Biol. Chem. **276:**40583–40590.
46. **Rajendra, R., D. Malegaonkar, P. Pungaliya, H. Marshall, Z. Rasheed, J. Brownell, L. F. Liu, S. Lutzker, A. Saleem, and E. H. Rubin.** 2004. Topors functions as an E3 ubiquitin ligase with specific E2 enzymes and ubiquitinates p53. J. Biol. Chem. **279:**36440–36444.
47. **Rogel, A., M. Popliker, C. G. Webb, and M. Oren.** 1985. p53 cellular tumor antigen: analysis of mRNA levels in normal adult tissues, embryos, and tumors. Mol. Cell. Biol. **5:**2851–2855.
48. **Sdek, P., H. Ying, D. L. Chang, W. Qiu, H. Zheng, R. Touitou, M. J. Allday, and Z. X. Xiao.** 2005. MDM2 promotes proteasome-dependent ubiquitin-independent degradation of retinoblastoma protein. Mol. Cell **20:**699–708.
49. **Shimizu, H., L. R. Burch, A. J. Smith, D. Dornan, M. Wallace, K. L. Ball, and T. R. Hupp.** 2002. The conformationally flexible S9–S10 linker region in the core domain of p53 contains a novel MDM2 binding site whose mutation increases ubiquitination of p53 *in vivo*. J. Biol. Chem. **277:**28446–28458.
50. **Shimizu, H., D. Saliba, M. Wallace, L. Finlan, P. R. Langridge-Smith, and T. R. Hupp.** 2006. Destabilizing missense mutations in the tumour suppressor protein p53 enhance its ubiquitination *in vitro* and *in vivo*. Biochem. J. **397:**355–367.
51. **Sigal, A., and V. Rotter.** 2000. Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome. Cancer Res. **60:**6788–6793.
52. **Soussi, T., C. Caron de Fromentel, and P. May.** 1990. Structural aspects of the p53 protein in relation to gene evolution. Oncogene **5:**945–952.
53. **Soussi, T., and G. Lozano.** 2005. p53 mutation heterogeneity in cancer. Biochem. Biophys. Res. Commun. **331:**834–842.
54. **Tang, J., L. K. Qu, J. Zhang, W. Wang, J. S. Michaelson, Y. Y. Degenhardt, W. S. El-Deiry, and X. Yang.** 2006. Critical role for Daxx in regulating Mdm2. Nat. Cell Biol. **8:**855–862.

55. **Uchida, C., S. Miwa, K. Kitagawa, T. Hattori, T. Isobe, S. Otani, T. Oda, H. Sugimura, T. Kamijo, K. Ookawa, H. Yasuda, and M. Kitagawa.** 2005. Enhanced Mdm2 activity inhibits pRB function via ubiquitin-dependent degradation. EMBO J. **24:**160–169.
56. **Uldrijan, S., W. J. Pannekoek, and K. H. Vousden.** 2007. An essential function of the extreme C-terminus of MDM2 can be provided by MDMX. EMBO J. **26:**102–112.
57. **Vassilev, L. T., B. T. Vu, B. Graves, D. Carvajal, F. Podlaski, Z. Filipovic, N. Kong, U. Kammlott, C. Lukacs, C. Klein, N. Fotouhi, and E. A. Liu.** 2004. *In vivo* activation of the p53 pathway by small-molecule antagonists of MDM2. Science **303:**844–848.
58. **Vousden, K. H.** 2002. Activation of the p53 tumor suppressor protein. Biochim. Biophys. Acta **1602:**47–59.
59. **Vousden, K. H.** 2006. Outcomes of p53 activation: spoilt for choice. J. Cell Sci. **119:**5015–5020.
60. **Wallace, M., E. Worrall, S. Pettersson, T. R. Hupp, and K. L. Ball.** 2006. Dual-site regulation of MDM2 E3-ubiquitin ligase activity. Mol. Cell **23:**251–263.
61. **Webley, K. M., A. J. Shorthouse, and J. A. Royds.** 2000. Effect of mutation and conformation on the function of p53 in colorectal cancer. J. Pathol. **191:**361–367.
62. **Weger, S., E. Hammer, and R. Heilbronn.** 2002. Topors, a p53 and topoisomerase I binding protein, interacts with the adeno-associated virus (AAV-2) Rep78/68 proteins and enhances AAV-2 gene expression. J. Gen. Virol. **83:**511–516.
63. **Whitesell, L., P. D. Sutphin, E. J. Pulcini, J. D. Martinez, and P. H. Cook.** 1998. The physical association of multiple molecular chaperone proteins with mutant p53 is altered by geldanamycin, an hsp90-binding agent. Mol. Cell. Biol. **18:**1517–1524.
64. **Wong, K. B., B. S. DeDecker, S. M. Freund, M. R. Proctor, M. Bycroft, and A. R. Fersht.** 1999. Hot-spot mutants of p53 core domain evince characteristic local structural changes. Proc. Natl. Acad. Sci. USA **96:**8438–8442.
65. **Wu, X., J. H. Bayle, D. Olson, and A. J. Levine.** 1993. The p53-mdm-2 autoregulatory feedback loop. Genes Dev. **7:**1126–1132.
66. **Yamasaki, S., N. Yagishita, T. Sasaki, M. Nakazawa, Y. Kato, T. Yamadera, E. Bae, S. Toriyama, R. Ikeda, L. Zhang, K. Fujitani, E. Yoo, K. Tsuchimochi, T. Ohta, N. Araya, H. Fujita, S. Aratani, K. Eguchi, S. Komiya, I. Maruyama, N. Higashi, M. Sato, H. Senoo, T. Ochi, S. Yokoyama, T. Amano, J. Kim, S. Gay, K. Nishioka, A. Hukamizu, K. Tanaka, and T. Nakajima.** 2006. Cytoplasmic destruction of p53 by the endoplasmic reticulum-resident ubiquitin ligase 'Synoviolin'. EMBO J. **26:**113–122.
67. **Yewdell, J. W., J. V. Gannon, and D. P. Lane.** 1986. Monoclonal antibody analysis of p53 expression in normal and transformed cells. J. Virol. **59:**444–452.
68. **Yu, G. W., S. Rudiger, D. Veprintsev, S. Freund, M. R. Fernandez-Fernandez, and A. R. Fersht.** 2006. The central region of HDM2 provides a second binding site for p53. Proc. Natl. Acad. Sci. USA **103:**1227–1232.
69. **Zhu, Q., J. Yao, G. Wani, M. A. Wani, and A. A. Wani.** 2001. Mdm2 mutant defective in binding p300 promotes ubiquitination but not degradation of p53: evidence for the role of p300 in integrating ubiquitination and proteolysis. J. Biol. Chem. **276:**29695–29701.