Ubiquitination and Degradation of Mutant p53

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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 at 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-
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), MCF-7/p53II 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 wild-type p53 and stably transfected with a construct encoding a p53 mutant were purchased from Chemicon and Calbiochem, respectively. Anti-Cdk4 antibodies (Calbiochem). Cop1-specific antibody was kindly provided by V. Dixit. ARF-Mdm2 and CHIP expression, predesigned pools of four siRNA oligonucleotides (SMARTpool; Dharmacon) were used. In the control experiments, nontargeting siRNA oligonucleotides were transfected twice with Mdm2-specific siRNA with HiPerfect reagent (QIAGEN) or Lipofectamine 2000 (Invitrogen). For the down-regulation of Cop1, ARF-BP1, and CHIP expression, cells were transfected twice with siRNA with 24 h between transfections.

Analysis of half-life of p53 by pulse-chase. The day before transfection, 10 μg p53/m2−/− 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 replaced with DMEM supplemented with 15 μg of methionine/liter and 24 μg 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 and degradation. Immunoprecipitation was carried out according to a method similar to one described previously (29). Twenty-four hours before transfection, 3 × 105 H1299 cells were seeded into 6-cm dishes. 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 μM 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 antibody Ab-1 (Calbiochem) or Ab-2 (Santa Cruz Biotechnology) and 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 μg MG132 for 24 h at 4°C. 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 200 μl of 10-fold concentrated sample buffer. Proteins from whole-cell extracts and immunoprecipitations were resolved by SDS-PAGE and analyzed by Western blotting with anti-HA antibody (Santa Cruz) and antibody anti-Mdm2 Ab-1 and Ab-2 antibodies (Calbiochem).

Analysis of half-life of p53 by pulse-chase. The day before transfection, 10 μg 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 replaced with DMEM supplemented with 15 μg of methionine/liter and 24 μg 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).
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).

**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 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
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 (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).
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

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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 p53 and p53 DBD mutant proteins were analyzed. p53<sup>−/−</sup> mdm2<sup>−/−</sup> (DKO) cells were transfected with plasmids encoding wild-type p53 (wt-p53) 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 [35S]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<sup>−/−</sup> mdm2<sup>−/−</sup> (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.

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
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 4 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. (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 DBD mutants along with an empty vector, wild-type Mdm2, or Mdm2 lacking the N-terminal p53 binding site (Δ58-89). (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).

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