Regulation of p53 Nuclear Export through Sequential Changes in Conformation and Ubiquitination* S

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Linghu Nie 1, Mark Sasaki 1, and Carl G. Maki 2
From the Department of Radiation and Cellular Oncology, University of Chicago, Chicago, Illinois 60637

Wild-type p53 is a conformationally labile protein that under-goes nuclear-cytoplasmic shuttling. MDM2-mediated ubiquitination promotes p53 nuclear export by exposing or activating a nuclear export signal (NES) in the C terminus of p53. We observed that cancer-derived p53s with a mutant (primary antibody 1620+/pAb240+) conformation localized in the cytoplasm to a greater extent and displayed increased susceptibility to ubiquitination than p53s with a more wild-type (primary antibody 1620+/pAb240−) conformation. The cytoplasmic localization of mutant p53s required the C-terminal NES and an intact ubiquitination pathway. Mutant p53 ubiquitination occurred at lysines in both the DNA-binding domain (DBD) and C terminus. Interestingly, Lys to Arg mutations that inhibited ubiquitination restored nuclear localization to mutant p53 but had no apparent effect on p53 conformation. Further studies revealed that wild-type p53, like mutant p53, is ubiquitinated by MDM2 in both the DBD and C terminus and that ubiquitination in both regions contributes to its nuclear export. MDM2 binding can induce a conformational change in wild-type p53, but this conformational change is insufficient to promote p53 nuclear export in the absence of MDM2 ubiquitination activity. Taken together, these results support a stepwise model for mutant and wild-type p53 nuclear export. In this model, the conformational change induced by either the cancer-derived mutation or MDM2 binding precedes p53 ubiquitination. The addition of ubiquitin to DBD and C-terminal lysines then promotes nuclear export via the C-terminal NES.

The tumor suppressor protein p53 is inactivated in the vast majority of human cancers, either through mutation, cytoplasmic sequestration, interaction with viral oncoproteins, or increased interactions with its negative regulator, MDM2 (1). It is therefore important to determine how p53 is normally regulated and how this regulation is altered in cancer. Wild-type p53 is a short lived protein and is expressed at low levels in most normal cells (2, 3). MDM2 is an E3 ubiquitin-protein ligase that can bind p53 and promote its rapid ubiquitin-mediated proteolysis (4, 5). Importantly, MDM2 is the product of a p53-inducible gene, thus establishing a negative feedback loop in which p53 increases expression of its own inhibitor (6, 7). The importance of MDM2 in regulating p53 has been revealed through genetic studies in which embryonic lethality in MDM2 knock-out mice is rescued by simultaneous knock-out of p53 (8). Recently, other ubiquitin-protein ligases have been identified that can also bind p53 and promote its degradation, including Pirh2, Cop1, and Chip (9–11). At least some of these (Pirh2 and Cop1) have been identified as p53-responsive gene products, similar to MDM2. The relative importance of these ligases and the context in which they may regulate p53 have not been fully clarified.

MDM2, in addition to promoting p53 degradation, can also affect the localization of p53. Two nuclear export signals (NES) have been identified in p53, one located within the C-terminal oligomerization domain and the other located in the N terminus (12, 13). MDM2 promoted wild-type p53 nuclear export in cells transiently co-expressing both proteins (14, 15). This effect required the C-terminal NES of p53 and was not observed with an MDM2 mutant that lacks its ability to promote p53 ubiquitination. It was also reported that mutation of the six C-terminal lysines in p53 inhibited its nuclear export by MDM2 (16, 17). These findings supported a model in which MDM2-mediated ubiquitination of one or more lysines in the p53 C terminus promotes p53 nuclear export by exposing or activating the C-terminal NES. In this regard, it is worth noting studies in which wild-type p53 that is sequestered in the cytoplasm of breast, liver, and neuroblastoma cancer cell lines shuttled back into the nucleus upon treatment with either MDM2 antisense oligonucleotides, or the nuclear export inhibitor leptomycin-B (12, 18, 19). These findings suggested cytoplasmic localization of p53 in these cancers is MDM2-dependent and results from excessive nuclear export.

Over 50% of human cancers harbor missense mutations in the p53 gene (20). These mutations are found almost exclusively in the DNA-binding domain (21, 22) and inhibit the ability of p53 to activate expression of its downstream target genes. Cancer-derived p53 mutations also affect the conformation and localization of p53 to varying extents. Changes in p53 conformation can be monitored by reactivity with conformation-specific antibodies that recognize p53 in either a wild-type (pAb1620) or mutant (pAb240) conformation (23, 24). Not-

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1 Both authors contributed equally to this work and should be considered as first authors.

2 To whom correspondence should be addressed: Dept. of Radiation and Cellular Oncology, the University of Chicago, 5841 S. Maryland Ave., MC1105 Rm. G06, Chicago, IL 60637. Tel.: 773-834-4391; E-mail: cmaki@rover.uchicago.edu.

The abbreviations used are: NES, nuclear export signal; DBD, DNA-binding domain; DAPI, 4,6-diamidino-2-phenylindole; pAb, primary antibody; TEV, tobacco etch virus; HA, hemagglutinin; WT, wild type; DKO, double knock-out; Ub, ubiquitin.

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withstanding changes in conformation, a number of other unique properties have been ascribed to mutant p53s that can distinguish them from the wild-type protein. Most obvious among these is the pronounced stability exhibited by mutant p53s. This stability may result from either association of mutant p53s with stabilizing complexes, some of which include Hsp90 (25), or from an inability of mutant p53s to activate expression of proteins like MDM2, PirH2, or Cop1 that could promote its degradation. Mutant p53s are also susceptible to various post-translational modifications to which wild-type p53 may be relatively resistant, such as ribosylation and ubiquitination (26, 27). Finally, a number of oncogenic and growth-promoting properties have been ascribed to mutant p53. Included among these is the ability to inhibit the transcriptional activity of wild-type p53, and the p53 family members p73 and p63, in a dominant-negative fashion (28). Such oncogenic properties may explain the selective retention of mutant p53s in cancer (29, 30).

In this study, we observed that certain cancer-derived p53s with a mutant (pAb1620−/pAb240+) conformation localized in the cytoplasm and displayed an increased susceptibility to ubiquitination. The cytoplasmic localization of mutant p53s depended on the C-terminal NES and an intact ubiquitination pathway. Ubiquitination of mutant p53 occurred at lysines in the DBD and the C terminus. Interestingly, Lys to Arg mutations in both regions contribute to its nuclear export. MDM2 binding induces a conformational change in wild-type p53, and the p53 family members p73 and p63, in a dominant-negative fashion (28). Such oncogenic properties may explain the selective retention of mutant p53s in cancer (29, 30).

**EXPERIMENTAL PROCEDURES**

Cell Culture and Transfections—Saos-2 (p53-null) and U2OS (p53 wild-type) are osteosarcoma cell lines. p53/MDM2 double knock-out (DKO) mouse embryo fibroblasts were from Rudy Alarcon (Stanford University). These cells were maintained at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 μg/ml penicillin and streptomycin. ts-20 cells were from Harvey Ozer (University of Medicine and Dentistry, New Jersey) and harbor a temperature-sensitive mutation in the E1 ubiquitin-activating enzyme system (31). ts-20 cells were maintained at 32 or 39 °C, as indicated in the text. Transfections were done using FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s protocol when cells were ~60% confluent. Briefly, cells were transfected with 0.5−1.0 μg of FLAG p53 DNA alone or with 1.0 μg of DNA encoding MDM2 and/or Myc-tagged ubiquitin (myc-Ub). Total DNA in each transfection was equalized by the addition of empty plasmid. 100 μl of serum and antibiotic-free Dulbecco’s modified Eagle’s medium were mixed with 3 μl of FuGENE 6, incubated at room temperature for 5 min, and then mixed with the appropriate DNAs. Following 30 min of incubation at room temperature, the mixture was added to cells to initiate transfection. Cell lysates were harvested 20−24 h later.

**Plasmid DNAs**—FLAG-tagged wild-type p53 has been described previously (32) and was from Zhi-min Yuan (Harvard School of Public Health). This DNA contains wild-type p53 sequences cloned into BamHI and XbaI sites downstream of the FLAG epitope. Myc-tagged ubiquitin DNA (33) was from Ron Kopito (Stanford University). DNAs encoding untagged p53 mutants V143A, R248W, and H179E were from Peter Howley (Harvard Medical School). DNAs encoding wild-type MDM2 and HA-tagged p53 C135Y were from Steve Grossman (University of Massachusetts Medical School). Untagged p53 mutant DNAs were amplified by PCR to contain a BamHI site at their N termini and an EcoRI site at their C termini and were then cloned into the BamHI and EcoRI sites downstream of the FLAG epitope. NES mutants of FLAG p53 C135Y and FLAG p53 V143A were generated using the QuikChange site-directed mutagenesis kit (Stratagene) with the following primer and its complement: 5′-GAGCTGAGTGGCCGCGAGCA-CAAGAGTCCCAGCTGCT-3′. Lysine to arginine mutants of FLAG p53 WT and FLAG p53 C135Y were also generated using the QuikChange mutagenesis kit using FLAG p53 (WT or C135Y) as the template and the following primers and their complements: for the K372R, K373R mutation, 5′-GAGCCAC-CTGAAGTGCTCCAGTCTACCTCC-3′; for the K370R mutation, 5′-CACTCCACCCACCTGAGGCAGAAGGCTCAAG-3′; for the K381R, K382R mutation, 5′-CTAC-CTCCGCGCATTAGGGGACTCAAGTGTCCG-3′; and for the K386R mutation, 5′-CTCATGTTCAGGAGACAGAGGAGGAG-3′. K387R mutation, 5′-GCCAGGCTGGGAGGGAGCCAAGGACGACG-3′; for the K351R mutation, 5′-CTTCTCCACCCGAGGAAGGAGAGCCTGGAG-3′; for the K305R mutation, 5′-GGAGCAGCAGCAGCACT-3′; for the K292R, K293R mutation, 5′-CTCCGAGGAGAGGAAGGCTCTTTCGAG-3′; for the K164R mutation, 5′-GCGCTCACTCCAGATGCTG-3′. 5′-GCCCTCAAAAGGATGTTTAC-3′; for the K120R mutation, 5′-GGAGACAGGAGGATGCTG-3′; for the K101R mutation, 5′-GATTAACCAGCTAC-3′; and for the K24R mutation, 5′-GACCTATGGAGACTA-3′. 5′-GTCTTCTCCACCCGAGGAGTCCAGAG-3′. These results suggested that conformational change could expose lysines in mutant p53 for ubiquitination, and ubiquitination of these lysines promotes nuclear export. Wild-type p53, like mutant p53, is ubiquitinated by MDM2 in both the DBD and C terminus, and ubiquitination in both regions contributes to its nuclear export. MDM2 binding induces a conformational change in wild-type p53, but this conformational change is insufficient to promote p53 nuclear export in the absence of MDM2 ubiquitination activity. Based on these results, we suggest a model in which MDM2-binding induces a conformational change in wild-type p53 that exposes lysines for ubiquitination. Ubiquitination, in turn, exposes the C-terminal NES for nuclear export.
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Immunoprecipitations, Immunoblotting, and TEV Cleavage—To harvest cell lysates, cells were rinsed with 2 ml of phosphate-buffered saline and then scraped into 500 μl of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, phenylmethylsulfonyl fluoride, leupeptin) and transferred to microcentrifuge tubes. The cells were then incubated on ice for 30 min with occasional vortexing and spun at 4 °C at 14,000 rpm in a microcentrifuge. For immunoprecipitations, the resulting supernatant was mixed for 1 h with 30 μl of protein A-agarose beads to pre-clear the lysate. The supernatant from this pre-clear was immunoprecipitated for 12–15 h at 4 °C with 2 μl of anti-FLAG polyclonal antibody (F-7145, Sigma) or 1.75 μl of p53 wild-type conformation-specific antibody (pAb1620, Ab-5, Calbiochem) or mutant conformation-specific antibody (pAb240, Ab-3, Calbiochem). Immunoprecipitations were incubated with 30 μl of protein A-agarose beads for 1 h, and the beads were isolated by centrifugation for 10 s at 13,000 rpm. For direct immunoblot analysis without TEV cleavage, the beads were washed twice with 1 ml of ice-cold lysis buffer and boiled for 10 min. For TEV cleavage, the beads were washed twice with 1 ml of ice-cold phosphate-buffered saline and once with ice-cold TEV buffer (50 mM Tris, pH 7.5, 0.5 mM EDTA, 1 mM dithiothreitol). The beads were then resuspended in 25 μl of TEV buffer, and 1 μl of TEV protease was added. The beads were rotated in the presence of TEV protease for 3 h at room temperature, and the beads were pelleted by centrifugation for 10 s at 13,000 rpm. The supernatant contained the TEV cleavage products and was boiled for 10 min. Products were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes for immunoblotting. Antibodies used in immunoblotting to detect p53 included the sheep polyclonal antibody Ab-7 (Oncogene Science), monoclonal p53 antibody 1801 (Oncogene Science), and the FLAG monoclonal antibody Ab-5 (Sigma). MDM2 antibody was SMP-14 (Santa Cruz Biotechnology).

Immunofluorescence—Immunofluorescence staining was carried out as described previously (34). Briefly, cells were transfected on glass coverslips in 6-well dishes. Cells were fixed FIGURE 1. Cancer-derived p53s with a mutant conformation display a largely cytoplasmic localization. A, U2OS cells were transfected with DNAs (1 μg) encoding the indicated FLAG-tagged p53s. p53 localization was monitored by immunofluorescence staining with an anti-FLAG antibody (red), and DNA was visualized by DAPI staining (blue). B, localization of the indicated p53 proteins was scored as either nuclear only or mostly nuclear (N, N > C), nuclear equal to cytoplasmic or mostly cytoplasmic (N = C, C > N). 150 cells were scored in three separate experiments. The graph shows the percent cells with the indicated p53 localization (± S.E.). C, cells were transfected with DNAs (1 μg each) encoding the indicated FLAG-tagged p53s. Transfected cell lysates were divided in half, immunoprecipitated (IP) with either wild-type (w; pAb1620) or mutant (m, pAb240) conformation-specific p53 antibodies, and examined by immunoblotting (IB) with anti-FLAG monoclonal antibody. The arrow indicates the FLAG-tagged p53 protein.
20–24 h later by incubation in 4% paraformaldehyde for 45 min. Cells were blocked by two 5-min incubations in the presence of 1% bovine serum albumin, 0.1% Triton X-100. FLAG p53s were detected with the anti-FLAG p53 polyclonal antibody F-7145 (Sigma) as the primary antibody, and with rhodamine-conjugated anti-rabbit antibody (The Jackson Laboratory) as the secondary antibody. DNA was stained with DAPI. Cells were visualized using a fluorescence microscope. Merged images showing FLAG p53 localization, and DNA stainings were obtained. The staining pattern for p53 was scored for 100–150 cells in two or three separate experiments.

RESULTS

Mutant p53s Are Cytoplasmic and Dependent on Their C-terminal NES and an Intact Ubiquitination Pathway—Cancer-associated mutations in p53 occur almost exclusively in the DNA-binding domain (DBD) and alter the conformation and localization of p53 to varying extents. p53 conformation can be monitored by assessing reactivity with the conformation-specific antibodies pAb1620 (wild-type specific) and pAb240 (mutant specific) (23, 24). In our first experiment, Saos-2 cells (p53 null) were transfected with DNAs encoding FLAG-tagged p53 that was either wild-type or harbored different cancer-associated mutations. Transfected cell lysates were then immunoprecipitated with wild-type (pAb1620) or mutant (pAb240) antibodies, followed by immunoblotting for p53. The relative amount of p53 immunoprecipitated by the wild-type or mutant-specific antibodies is an indication of p53 conformation. As shown in Fig. 1C, wild-type (WT) p53 and p53 R248W displayed a mostly wild-type conformation, and p53 C135Y, H179E, and V143A were all mostly mutant in their conformation. Next, we monitored localization of the different p53 proteins by immunofluorescence staining. As shown in Fig. 1, A and B, wild-type p53 and p53 R248W displayed a mostly nuclear localization, whereas p53 C135Y, V143A, and H179E localized throughout the nucleus and cytoplasm in most cells in which they were expressed. These results support a correlation between the conformation and localization of p53. Specifically, p53s with a mostly mutant conformation (such as V143A, C135Y, and H179E) localize in the cytoplasm to a greater extent than p53s with a more wild-type conformation (such as R248W).

Wild-type p53 contains an NES in its C terminus that is required for MDM2-dependent nuclear export (14, 15). We reasoned that if cytoplasmic localization of cancer-derived p53 mutants involves nuclear export, then mutation of this NES would cause the mutants to assume a more nuclear localization. To test this, we mutated the C-terminal NES in p53 C135Y and p53 V143A and monitored their localization in transfected cells. As shown in Fig. 2, A and B, nonmutated p53 C135Y and p53 V143A displayed largely cytoplasmic localizations, whereas the p53 C135Y NES and p53 V143A NES mutants were re-localized almost completely to the nucleus. This indicates that cytoplasmic localization of these mutants requires an intact NES in the p53 C terminus. Importantly, the NES mutations had no apparent effect on conformation of p53 C135Y and p53 V143A, at least to the extent the wild-type and mutant-specific antibodies can recognize changes in p53 conformation (Fig. 2C). This suggests that nuclear accumulation of the NES mutants was not due to a change in their conformation. In total, these results suggest cytoplasmic localization of mutant p53s involves nuclear export via the p53 C-terminal NES. It should also be noted that the NES mutant of p53 C135Y was still ubiquitinated in cells when co-expressed with myc-Ub (Fig. 4B), indicating the nuclear accumulation of p53 C135Y NES was not due to an inability to be ubiquitinated.

MDM2-dependent nuclear export of wild-type p53 requires an intact ubiquitination pathway. Support for this comes from studies in which p53 nuclear export by MDM2 was inhibited at the nonpermissive temperature in ts-20 cells (15), which harbor a temperature-sensitive mutation in the ubiquitin-activating enzyme. These cells maintain ubiquitination activity at 32 °C but lose ubiquitination activity at 39 °C (35). To test whether an intact ubiquitination system is required for mutant p53 nuclear export, we monitored p53 C135Y localization in ts-20 cells at 32 °C and nonpermissive 39 °C. We also monitored wild-type p53 nuclear export at both temperatures as a control for the experiment. As shown in Fig. 3A, wild-type p53 was mostly nuclear when expressed alone, but re-localized to the cytoplasm (was exported) when co-expressed with MDM2 in ts-20 cells that were maintained at 32 °C. In contrast, wild-type p53
remained largely nuclear when expressed alone or with MDM2 in ts-20 cells that were grown at 39 °C to inhibit ubiquitination activity. This is consistent with previous findings that ubiquitination activity is required for MDM2 to promote p53 nuclear export (15). Mutant p53 (C135Y) was mostly cytoplasmic in ts-20 cells at 32 °C, but it accumulated in the nucleus in ts-20 cells at 39 °C (Fig. 3B). This indicates that nuclear export of mutant p53 (C135Y) also depends, at least in part, on an intact ubiquitination pathway. p53 C135Y displayed a mostly cytoplasmic localization at both 32 and 39 °C in U2OS cells, indicating that nuclear accumulation of p53 C135Y at 39 °C is only seen in cells (ts-20) where ubiquitination activity can be inhibited at elevated temperature.

Mutant Conformation p53s Display Increased Susceptibility to Ubiquitination—Nuclear export of wild-type p53 is thought to result from ubiquitination of p53 C-terminal lysines and exposure of the C-terminal NES (16, 17, 36). Since mutant p53s undergo nuclear export via their C-terminal NES, we wished to examine whether mutant p53s are ubiquitinated in their C terminus and whether this ubiquitination contributes to their nuclear export. To this end, cells were transfected with DNAs encoding FLAG-tagged p53s either alone or with DNA encoding myc-Ub DNA. Lysates were immunoprecipitated (IP) with an anti-Myc antibody, followed by immunoblotting (IB) with a FLAG monoclonal antibody. The ladder of bands are ubiquitin-modified forms of each p53 C135Y protein. C. p53/MDM2 DKO cells were transfected with DNA encoding FLAG-p53 C135Y and myc-Ub, as indicated. Lysates were immunoprecipitated with a FLAG polyclonal antibody, followed by immunoblotting with a FLAG monoclonal antibody. The arrows indicate ubiquitin-modified p53 C135Y.

FIGURE 4. Cancer-derived p53 mutants display increased susceptibility to ubiquitination. A. U2OS cells were transfected with DNA encoding the indicated FLAG p53 alone, or with DNA encoding myc-Ub (1 μg each). Lysates were examined by immunoblotting with an anti-FLAG antibody. The position of non ubiquitinated p53 is indicated. Arrows (right) indicate ubiquitin-modified forms of mutant p53s that were evident with myc-Ub co-expression. B. cells were transfected with DNAs encoding p53 C135Y, p53 C135Y 342N, or p53 C135Y NES DNA either alone or with myc-Ub DNA. Lysates were immunoprecipitated (IP) with an anti-Myc antibody, followed by immunoblotting (IB) with a FLAG monoclonal antibody. The position of non ubiquitinated p53 is indicated. Arrows (right) indicate ubiquitin-modified forms of mutant p53s that were evident with myc-Ub co-expression. C. p53/MDM2 DKO cells were transfected with DNA encoding FLAG-p53 C135Y and myc-Ub, as indicated. Lysates were immunoprecipitated with a FLAG polyclonal antibody, followed by immunoblotting with a FLAG monoclonal antibody. The arrows indicate ubiquitin-modified p53 C135Y.

FIGURE 3. Nuclear export of mutant p53s requires an intact ubiquitination system. A, ts-20 cells were grown at 37 °C and transfected with DNAs encoding FLAG-tagged WT p53 and MDM2 (1 μg each). 3–4 h after transfection, the cells were switched to either 32 or 39 °C for 20 h. p53 localization was monitored by immunofluorescence staining in cells in which it was co-expressed with MDM2, and p53 localization was scored. The graph shows the percent cells with the indicated p53 localization. Average results from two separate experiments are shown. MDM2 displayed a nuclear only localization in the majority of cells. Cells in which MDM2 was not completely nuclear were excluded from the analysis. B, ts-20 cells and U2OS cells were transfected with DNA encoding FLAG-tagged p53 (C135Y) (1 μg). The cells were then either maintained at 32 °C or shifted to 39 °C for 20 h. p53 C135Y localization was monitored by immunofluorescence staining with an anti-FLAG polyclonal antibody, and p53 C135Y localization was scored. The graph shows the percent cells with the indicated p53 C135Y localization. Average results from two separate experiments are shown.

FIGURE 4. Cancer-derived p53 mutants display increased susceptibility to ubiquitination. A. U2OS cells were transfected with DNA encoding the indicated FLAG p53 alone, or with DNA encoding myc-Ub (1 μg each). Lysates were examined by immunoblotting with an anti-FLAG antibody. The position of non ubiquitinated p53 is indicated. Arrows (right) indicate ubiquitin-modified forms of mutant p53s that were evident with myc-Ub co-expression. B. cells were transfected with DNAs encoding p53 C135Y, p53 C135Y 342N, or p53 C135Y NES DNA either alone or with myc-Ub DNA. Lysates were immunoprecipitated (IP) with an anti-Myc antibody, followed by immunoblotting (IB) with a FLAG monoclonal antibody. The ladder of bands are ubiquitin-modified forms of each p53 C135Y protein. C. p53/MDM2 DKO cells were transfected with DNA encoding FLAG-p53 C135Y and myc-Ub, as indicated. Lysates were immunoprecipitated with a FLAG polyclonal antibody, followed by immunoblotting with a FLAG monoclonal antibody. The arrows indicate ubiquitin-modified p53 C135Y.

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To test whether mutant p53 ubiquitination requires MDM2, we first deleted the N-terminal 42 amino acids, including the MDM2-binding domain, from p53 C135Y and tested whether the resulting deletion mutant was ubiquitinated. As shown in Fig. 4B, FLAG-tagged p53 C135Y was highly ubiquitinated when co-expressed with myc-Ub, and the N-terminal deletion mutant (C135Y Δ42N) was also ubiquitinated, although obviously less well. This suggests the N terminus and MDM2-binding domain may contribute to mutant p53 ubiquitination but are not absolutely required. Next, we tested whether p53 C135Y could be ubiquitinated in p53 and MDM2 DKO cells. As shown in Fig. 4C, p53 C135Y was modified by myc-Ub in p53/MDM2 DKO cells, although to a lower extent. Based on these results we conclude that the ubiquitination of mutant p53s can occur in an MDM2-independent manner.

Lysines in the C Terminus and DBD Are Ubiquitinated in Mutant p53—p53 contains 20 lysine residues throughout its sequence, including six clustered in the C terminus and five clustered in the DNA-binding domain (Fig. 5A, DBD). The six C-terminal lysines are thought to be the major sites for MDM2-mediated ubiquitination and nuclear export of wild-type p53 (16, 17). To identify lysines in mutant p53 that are targeted for ubiquitination, various lysine to arginine mutations were introduced into FLAG p53 C135Y. Ubiquitination of the resulting proteins was assessed in transfected cells when expressed alone or with myc-Ub. This involved immunoprecipitation of transfected p53 using a FLAG antibody, followed by immunoblotting with a Myc antibody. As shown in Fig. 5B, nonmutated p53 C135Y (0KR) was ubiquitinated when expressed with myc-Ub alone, as evidenced by a high molecular weight smear and ladder of bands detected by the Myc antibody. Conversion of the 14 lysines to arginine (14KR) in the C-terminal one-third of p53 C135Y diminished but did not completely inhibit its ubiquitination. This suggested p53 C135Y ubiquitination may occur at one or more of these 14 lysines but that ubiquitination can also occur within the remaining N-terminal 6 lysines in the 14KR mutant. We suspected ubiquitination in the N terminus probably occurs at lysines in the DBD, since this is the region whose conformation is altered by cancer-derived mutations. To test this, we monitored ubiquitination of p53 C135Y in which the five DBD lysines were converted to arginine (N5KR). As shown in Fig. 5C, ubiquitination was diminished in the p53 C135Y N5KR mutant, consistent with these lysines being sites for mutant p53 ubiquitination. Conversion of the six C-terminal lysines to arginine (C6KR) also diminished C135Y ubiquitination, and combining the N5KR and C6KR mutations in p53 C135Y completely inhibited its ubiquitination, similar to the 20KR mutant. In total, these results indicate lysines in the C terminus and DBD are ubiquitinated in mutant p53.

Lysines in the C Terminus and DBD Contribute to Mutant p53 Nuclear Export—If ubiquitination contributes to mutant p53 nuclear export, then mutations that block this ubiquitination should cause the mutant p53 to accumulate in the nucleus. To test this, we monitored localization of p53 C135Y and the N5KR and C6KR mutants. As shown in Fig. 6, A and B, nonmutated p53 C135Y with all lysines intact (0KR) displayed a largely cytoplasmic localization, consistent with results presented ear-

FIGURE 5. Lysines in the DNA-binding domain and C terminus of mutant p53 are ubiquitinated. A, schematic of FLAG-tagged p53 C135Y DNA. The position of the 20 lysines (K) are indicated, including the six lysines clustered in the C terminus and the five lysines clustered in the N-terminal half of the DNA-binding domain. The lines above indicate the position of the various lysine (K) to arginine (R) mutations. Oligo, oligonucleotide. B, U2OS cells were transfected with the indicated p53 C135Y DNAs alone or with myc-Ub. Transfected cell lysates were immunoprecipitated with a FLAG polyclonal antibody, followed by immunoblotting with a Myc antibody. Conversion of the resulting proteins was assessed in transfected p53 C135Y using a FLAG antibody, followed by immunoblotting with a Myc antibody (FLAG IP/Myc IB). A ladder of bands (indicated by arrows) represent ubiquitination of the FLAG-tagged p53 proteins. Note 14KR was less ubiquitinated than 0KR. C, U2OS cells were transfected with the indicated p53 C135Y DNAs alone or with myc-Ub. Transfected cell lysates were immunoprecipitated with a FLAG polyclonal antibody, followed by immunoblotting with a Myc antibody (FLAG IP/Myc IB). Note the N5KR and 6KR mutants were less ubiquitinated than 0KR, and the N5KR+6KR and 20KR mutants were not obviously ubiquitinated.
lier. In contrast, both the N5KR and C6KR mutants were re-localized to the nucleus, and the double mutant (C6KR,N5KR) displayed an even greater nuclear localization than the N5KR or C6KR single mutants. These results suggest that ubiquitination of DBD and C-terminal lysines contributes to the cytoplasmic localization (nuclear export) of mutant p53 (C135Y). Importantly, the N5KR and C6KR mutants still displayed a mutant conformation (Fig. 6C), despite their apparent resistance to nuclear export. Taken together, the results suggest that conformational change induced by the p53 mutation renders p53 susceptible to ubiquitination in both the DBD and C terminus. Although less nuclear than either WT p53 or p53 C6KR, nonetheless, p53 N5KR was only modestly re-localized to the cytoplasm when expressed with MDMP, suggesting DBD lysine ubiquitination could contribute to WT p53 nuclear export. Consistent with this possibility, p53-(Δ101–195) was also resistant to MDMP-dependent nuclear export, displaying a mostly nuclear localization when expressed alone or with MDMP. Finally, a double mutant FLAG p53 (Δ101–195, C6KR) was mostly nuclear when expressed alone or with MDMP, demonstrating that it is resistant to MDMP-mediated nuclear export. These mutants also displayed varying degrees of

**FIGURE 6. Lysine to arginine mutations affect mutant p53 localization but do not affect conformation.**

A, U2OS cells were transfected with DNAs encoding FLAG-tagged p53 C135Y or the indicated lysine to arginine mutants. p53 localization (red) was monitored by immunofluorescence staining with an anti-FLAG polyclonal antibody, and DNA (blue) visualized by DAPI staining. Representative pictures are shown. B, p53 localization was scored in 100 cells in three separate experiments, as in Figs. 2 and 3. The graph shows the percent cells with the indicated p53 localization (± S.E.). C, cells were transfected with the indicated FLAG-tagged p53 C135Y DNAs. Transfected cell lysates were divided in half, immunoprecipitated (IP) with either wild-type (w, pAb1620) or mutant (m, pAb240) conformation-specific p53 antibodies, and examined by immunoblotting with an anti-FLAG monoclonal antibody. The arrow indicates the FLAG-tagged p53 protein. Ab indicates detection of the antibody heavy chain used in the immunoprecipitation. cont, control.
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In summary, both DBD and C-terminal lysines in WT p53 are ubiquitinated by MDM2 (supplemental Figs. 1 and 2), and these lysines are also necessary for MDM2-mediated nuclear export.

p53 Conformational Change Caused by MDM2 Binding Is Insufficient to Promote Nuclear Export in the Absence of MDM2 Ubiquitination Activity—MDM2 binding can induce a conformational change in WT p53, evidenced by exposure of the pAb240 epitope (40). This effect does not require the MDM2 RING domain and p53 ubiquitination. We tested whether the conformational change induced by MDM2 binding was sufficient to promote p53 nuclear export. First, cells were co-transfected with equal amounts (1 μg each) of DNA encoding the indicated FLAG-tagged WT p53 and MDM2. Immunoblotting showed that p53 levels were only slightly decreased when expressed with MDM2 under these conditions (Fig. 8A). Next, we monitored the localization of the transfected cell lysates. As shown in Fig. 8B, wild-type p53 had a mostly wild-type conformation when expressed alone. However, co-expression with MDM2 WT caused an accumulation of p53 in a mutant conformation (recognized by the mutant conformation-specific antibody). This effect required MDM2 binding to p53 since MDM2 Δp53BD, which lacks the N-terminal p53-binding site in MDM2, was unable to promote an accumulation of p53 with a mutant conformation. In contrast, the MDM2 RING finger domain and ubiquitination activity were not required for this effect since co-expression with MDM2 ΔRING caused an accumulation of p53 in a mutant conformation similar to MDM2 WT. These results are consistent with reports that MDM2 binding can induce a conformational change in p53 that is independent of its ubiquitination activity (see Refs. 38 and 40). Finally, we monitored p53 nuclear export when expressed with either MDM2 WT, MDM2 Δp53BD, or MDM2 ΔRING. As shown in Fig. 8C, FLAG p53 was mostly nuclear when expressed alone, but it was exported and re-localized to the cytoplasm when co-expressed with MDM2 WT. In contrast, FLAG p53 remained nuclear when expressed with either MDM2 Δp53BD or MDM2 ΔRING. Thus, MDM2 binding can induce a conformational change in wild-type p53, but this change is not sufficient to promote nuclear export in the absence of MDM2 ubiquitination activity.

DISCUSSION

Nuclear cytoplasmic shuttling has emerged as an important determinant of p53 activity. It has been known for several years that MDM2 can promote the ubiquitination of p53 and its nuclear export. Still the details of how p53 nuclear export is regulated remain largely unknown. MDM2-mediated nuclear export of p53 is believed to require ubiquitination of one or more lysines located in a cluster of six lysines in the extreme C terminus of p53 (the six lysines are clustered between residues 370 and 386). This is based on reports that mutation of the six C-terminal lysines in p53 blocked both its ubiquitination by MDM2 and its nuclear export (16, 17). Further support came from Gu and co-workers (36) who reported that a p53-ubiquitin fusion protein, thought to mimic p53 ubiquitinated in its C terminus, displayed a completely cytoplasmic localization due to nuclear export in MDM2-null cells. p53 nuclear export by MDM2 also requires the C-terminal NES, which is located adjacent to p53 C-terminal sequences between residues 345 and 355 (12, 14, 15). It is speculated that the addition of ubiquitin to the C-terminal lysines might disrupt adjacent sequences, causing exposure or activation of the C-terminal NES and nuclear export.
Regulation of p53 Nuclear Export

Cancer-derived p53s Undergo Ubiquitin-dependent Nuclear Export—Certain cancer-derived p53s display increased susceptibility to ubiquitination and localize in the cytoplasm to varying extents. We observed that cancer-derived p53s with a mutant (pAb1620+/pAb240−) conformation localized in the cytoplasm to a greater extent than p53s with a more wild-type (pAb1620+/pAb240+) conformation. Mutation of the C-terminal NES restored nuclear localization to mutant p53s, suggesting their cytoplasmic localization results from nuclear export. Consistent with this, Akakura et al. (39) reported that a temperature-sensitive p53 mutant that normally localizes in the cytoplasm at high temperature was re-localized in the nucleus at high temperature in cells treated with the nuclear export inhibitor leptomycin B. These results are consistent with ours and further indicate that cytoplasmic localization of mutant p53 results from nuclear export. p53s with a mutant (pAb1620−/pAb240+) conformation were also more susceptible to ubiquitination than p53s with a more wild-type (pAb1620+/pAb240−) conformation. Two approaches were taken to address whether ubiquitination contributes to mutant p53 nuclear export. First, we monitored nuclear export of wild-type and mutant p53 in cells that harbor a temperature-sensitive ubiquitin-activating enzyme. Wild-type p53 nuclear export by MDM2 was inhibited in these cells when ubiquitination activity was blocked by the switch to high temperature, consistent with previous results (15). Mutant p53 also re-localized to the nucleus when ubiquitination activity was inhibited in these cells, indicating that nuclear export of mutant p53 depends on an intact ubiquitination pathway. Second, we carried out mutation analyses to map the lysines in mutant p53 that are ubiquitinated. These studies showed that lysines in the DBD and C terminus are ubiquitinated in mutant p53. The recognition of ubiquitination sites outside the C terminus is consistent with studies from Hupp and co-workers (27) in which another p53 mutant (F270A) was highly susceptible to ubiquitination at sites other than those in the C terminus. Mutation of DBD and/or C-terminal lysines that blocked their ubiquitination caused re-localization of mutant p53 (C135Y) to the nucleus.
This is also consistent with ubiquitination contributing to mutant p53 nuclear export. Importantly, mutant p53 with the DBD or C-terminal lysines mutated remained mutant (pAb240 reactive) in their conformation. Thus, conformational change alone is insufficient for nuclear export of mutant p53 in the absence of DBD and C-terminal lysines. In summary, our studies with mutant p53 suggest that conformational change (caused by the cancer-derived p53 mutation) renders mutant p53 susceptible to ubiquitination in both the DBD and C terminus. This ubiquitination appears necessary for efficient nuclear export of mutant p53 via the C-terminal NES (Fig. 9).

A Model for p53 Nuclear Export—Wild-type p53 undergoes ubiquitin-dependent nuclear export when expressed with MDM2, and we wished to determine whether our findings for mutant p53 nuclear export could be extended to the wild-type protein. Using cleavable forms of wild-type p53, we found that MDM2 ubiquitinates wild-type p53 in both the DBD and the C terminus (supplemental Figs. 1 and 2). Our studies are consistent with previous reports that MDM2 can ubiquitinate DBD lysines (37), in addition to those in the C terminus. Mutation of DBD and/or C-terminal lysines that blocked their ubiquitination by MDM2 also blocked p53 nuclear export. Thus, wild-type p53, like mutant p53, requires DBD and C-terminal lysines for nuclear export. MDM2 binding can induce a conformational change in wild-type p53, evidenced by exposure of the pAb240 epitope. This conformational change does not require the MDM2 RING finger and ubiquitination activity. Importantly, WT MDM2 and MDM2 ∆RING could both induce a conformational change in wild-type p53, but only WT MDM2 could promote p53 nuclear export. This indicates that conformational change alone is sufficient for nuclear export of wild-type p53 in the absence of MDM2 ubiquitination activity. In combination, these results suggest that wild-type p53 and mutant p53 nuclear export occur through similar mechanisms that involve both conformational change and ubiquitination (Fig. 9). In the case of wild-type p53, conformational change is induced by MDM2 binding and precedes p53 ubiquitination in the DBD and C terminus. Ubiquitination in both the DBD and C terminus is necessary for efficient nuclear export of wild-type p53 via the C-terminal NES.

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