MOLECULAR AND CELLULAR BIOLOGY, Feb. 2007, p. 1056–1068 Vol. 27, No. 3
0270-7306/07/$08.00+0 doi:10.1128/MCB.01307-06

Cancer-Associated Mutations in the MDM2 Zinc Finger Domain Disrupt Ribosomal Protein Interaction and Attenuate MDM2-Induced p53 Degradation▽

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Received 17 July 2006/Returned for modification 20 August 2006/Accepted 1 November 2006

The p53-inhibitory function of the oncoprotein MDM2 is regulated by a number of MDM2-binding proteins, including ARF and ribosomal proteins L5, L11, and L23, which bind the central acidic domain of MDM2 and inhibit its E3 ubiquitin ligase activity. Various human cancer-associated MDM2 alterations targeting the central acidic domain have been reported, yet the functional significance of these mutations in tumor development has remained unclear. Here, we show that cancer-associated missense mutations targeting MDM2's central zinc finger disrupt the interaction of MDM2 with L5 and L11. We found that the zinc finger mutant MDM2 is impaired in undergoing nuclear export and proteasomal degradation as well as in promoting p53 degradation, yet retains the function of suppressing p53 transcriptional activity. Unlike the wild-type MDM2, whose p53-suppressive activity can be inhibited by L11, the MDM2 zinc finger mutant escapes L11 inhibition. Hence, the MDM2 central zinc finger plays a critical role in mediating MDM2's interaction with ribosomal proteins and its ability to degrade p53, and these roles are disrupted by human cancer-associated *MDM2* mutations.

The mammalian p53 transcription factor mediates a major tumor suppression pathway that is negatively controlled by the proto-oncoprotein MDM2 (HDM2 in humans; henceforth denoted MDM2) and is altered in most, if not all, human cancers. The gene for mouse Mdm2 (murine double minute 2) was originally identified in a spontaneously transformed mouse BALB/c cell line (13). The Mdm2 protein was found to be responsible for transformation of NIH 3T3 and Rat2 cells when overexpressed (13), and this transforming function is believed to stem from its ability to bind with and inhibit the transactivation activity of p53 (39). Subsequently, the *HDM2* gene, the human homologue of *Mdm2*, was found to be amplified in over one-third of those human sarcomas that still retain wild-type p53 (41), suggesting that overexpression of MDM2 could be a common mechanism by which cells inactivate p53. Mice with targeted deletion of the *Mdm2* gene die during early embryonic development, and this lethality can be rescued by concomitant deletion of *p53*, indicating that a major in vivo function of MDM2 is to keep p53 activity in check (27, 33).

It is believed that MDM2 controls p53 through two mechanisms: inhibition of the transcriptional activity of p53 (39) and promotion of p53 ubiquitination and degradation (18, 29). Mdm2 binds to and masks the N-terminal transactivation domain of p53 by directly interfering with the interaction between p53 and the basal transcriptional machinery (42, 54). Mdm2 belongs to a large family of RING finger ubiquitin ligases (25). Studies have demonstrated that Mdm2 is a ubiquitin ligase (19) and that the ubiquitin ligase activity of Mdm2 is responsible for degradation of p53 both in vitro (14, 20) and in transfected cells (14). MDM2-mediated p53 degradation also depends on its ability to promote p53 nuclear export (46). Mutation of a nuclear export signal (NES) in MDM2 abolishes its ability to shuttle p53 to the cytoplasm for degradation (46). Similarly, blocking CRM-1 mediated nuclear export of NES-containing proteins with leptomycin B leads to nuclear accumulation and increased steady-state levels of p53 and MDM2 (15, 48). These findings suggest that, whether p53 shuttles out of the nucleus autonomously (53, 60) or in an MDM2-dependent manner (5, 16, 48), nuclear export of both MDM2 and p53 appears to be necessary for MDM2-targeted p53 degradation in the cytoplasm (reviewed in reference 61). Adding further complexity, enforced expression of MDM2 can also promote degradation of p53 in the nucleus, provided p53 and MDM2 are both in the same cellular compartment (57). It was demonstrated that low levels of MDM2 mediate p53 monoubiquitination and preferentially target p53 for export to the cytoplasm, whereas high levels of MDM2 lead to p53 polyubiquitination and degradation in both the nucleus and the cytoplasm (30). Recent reports indicate that the central acidic domain of MDM2 is important in controlling p53 activity (34, 55). Indeed, this domain was shown to be required for p53 ubiquitination and degradation (2, 37).

MDM2 interacts with p14ARF/p19Arf (ARF thereafter), and this interaction inhibits MDM2 and stabilizes and activates

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▽ Published ahead of print on 20 November 2006.

p53 (28, 44, 49, 62). Besides ARF, many other proteins have been identified to interact with MDM2 (reviewed in reference 23), including the retinoblastoma protein pRb (56) and the transcription factor E2F1 (36), both of which are prominent regulators of the cell cycle, suggesting that MDM2 may play a role in cell cycle regulation. MDM2 also interacts with ribosomal proteins L5 (9, 35), L11 (31, 59), and L23 (10, 24), indicating that Mdm2 is involved in regulating ribosomal biogenesis and cell growth (4). Concomitant p53 mutation and MDM2 overexpression within the same tumor have been found in a small fraction of clinical human cancers (6, 32), suggesting that MDM2 has a p53-independent tumorigenic function. Indeed, mice overexpressing Mdm2 in a p53-null background have a higher incidence of sarcomas than do p53-null mice, suggesting that a mechanism other than p53 inactivation exists for MDM2 to contribute to tumor development (26). In addition to genomic amplification and overexpression of MDM2, mutations within the *MDM2* gene have been reported in several types of human cancers (47, 51). Intriguingly, many of the mutant MDM2-containing cancers retain wild-type p53 (43). Because previous studies focused mostly on MDM2 gene amplification and protein overexpression, the prevalence of MDM2 mutations in human cancer is unknown and the functional significance of these mutations has not been characterized. In this study, we focus on several human cancer-derived MDM2 mutations described previously. We found that some of the mutations that target the central zinc finger of MDM2 can specifically disrupt the interaction of MDM2 with L11 and L5. These MDM2 mutants retain full p53-suppressive function while escaping inhibition by ribosomal protein L11. This study provides a potential mechanistic explanation for human cancer-derived mutations targeting the central zinc finger domain of MDM2.

MATERIALS AND METHODS

Plasmids. The cytomegalovirus (CMV) plasmid construct CMV-p53, pCDNA3-Myc-MDM2, CMV-MDM2, and deletion mutants thereof were described elsewhere (21, 24, 59). Mutant pCDNA3-MDM2^{C305F}, CMV-MDM2^{C305F}, and CMV-MDM2^{C308Y} constructs were generated by PCR-mediated site-directed mutagenesis with the QuikChange mutagenesis kit (Stratagene) and verified by DNA sequencing.

Cell culture and transfection. U2OS (p53-positive) osteosarcoma, H1299 (p53 negative) lung carcinoma, Saos-2 (p53 negative) osteosarcoma, HeLa (wild-type p53, human papillomavirus E6 [HPV-E6] positive), and *MDM2*$^{-/-}$; *p53*$^{-/-}$ (2KO) mouse embryonic fibroblast (MEF) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), L-Glu, and penicillin-streptomycin in 5% CO_2 using a humidified incubator. Cell transfections were carried out using the FuGENE 6 reagent (Roche).

Adenovirus and infection. Adenoviruses expressing wild-type or mutant MDM2^{C305F} were produced by subcloning full-length MDM2 into a transition vector, pCR259, followed by overlap recombination. For adenovirus infection, cells were infected with adenovirus in DMEM supplemented with 0.1% fetal bovine serum (FBS) and incubated for 2 h in a 37°C incubator with 5% CO_2. Cells were then washed with prewarmed phosphate-buffered saline (PBS) and replenished with fresh DMEM supplemented with 10% FBS.

Antibodies, immunofluorescence, and heterokaryon assay. Indirect immunofluorescence and the heterokaryon assay have been described previously (22). Immunostained cells were analyzed using an Olympus IX-81 microscope fitted with a SPOT camera and software. To generate antihuman ribosomal protein L5 antiserum, an N-terminally derived L5 peptide (VIQDKNYNTPKYRMC) was conjugated to keyhole limpet hemocyanin and used for rabbit immunization. Subsequent purification of the antiserum was done with the Sulfolink kit (Pierce). Generation of purified antibodies toward human ribosomal proteins L11 and L23 has been described earlier (24, 59). Rabbit polyclonal anti-p21 and rabbit anti-Myc were kindly provided by Yue Xiong. Anti-mouse LYAR antibody (50) was a gift from Lishan Su. The following antibodies were commercially purchased: rabbit polyclonal antibody to human p53 (FL393; Santa Cruz), rabbit polyclonal antibody to human MDM2 (N-20; Santa Cruz), p53 monoclonal antibody DO.1 (Neomarkers), monoclonal anti-MDM2 antibody SMP14 (Neomarkers) and 4B11 (UNC Tissue Culture and Molecular Biology Support Facility), mouse antihemagglutinin (HA) (clone 12CA5; Boehringer Mannheim), mouse monoclonal (PC10) anti-PCNA (Neomarkers), mouse antiactin (MAB1501; Chemicon International), mouse antitubulin (Neomarkers), and rabbit polyclonal anti-green fluorescent protein (GFP) (Research Diagnostics). For immunofluorescence experiments, the rhodamine red-, Cy2-, fluorescein isothiocyanate-, and 7-amino-4-methylcoumarin-3-acetic acid (AMCA)-conjugated secondary antibodies were purchased commercially (Jackson ImmunoResearch Laboratories).

Luciferase assay and protein half-life measurements. Luciferase activity was assayed using the Promega dual-luciferase assay kit as described previously (59). To measure p53 and MDM2 half-lives, protein synthesis was blocked by addition of cycloheximide (50 μg/ml) at the indicated time points, and the levels of p53 and MDM2 proteins were analyzed by immunoblotting. Procedures for immunoprecipitation and immunoblotting have been described previously (12).

In vivo ubiquitination assay. To detect ubiquitinated forms of p53 in vivo, H1299 cells were cotransfected with plasmids encoding wild-type or mutant MDM2 together with p53 as indicated in the text and cultured for an additional 20 h. After 4 h of treatment with 10 μM MG132, the cells were lysed directly in hot sodium dodecyl sulfate (SDS) sample buffer and MDM2-mediated p53 ubiquitination was assessed by Western blotting with antibodies against p53. In separate experiments, cells were cotransfected with MDM2 and p53 plasmids together with a plasmid expressing HA-tagged ubiquitin (HA-Ub) for 20 h. Cells were then treated with 10 μM MG132 for 4 h prior to harvest and lysed in 1% SDS lysis buffer followed by boiling for 10 min. The immunoprecipitation (IP)-Western blotting assay was carried out as described above.

RESULTS

Human cancer-derived mutations in the MDM2 central zinc finger disrupt L11 binding. Sequence alignment of MDM2 (eight mammals, frog, and zebra fish) revealed three highly conserved areas approximately at residues 20 to 110 (numbers according to human MDM2), 240 to 330, and 440 to 490 (Fig. 1A). The N-terminal conserved domain is believed to be important for p53 binding; the C-terminal conserved domain contains a RING finger that is essential for MDM2's E3 ubiquitin ligase function (14, 20). The function of the central acidic domain, including a highly conserved C4 zinc finger, is not fully understood. Previous studies have mapped a minimal domain around residues 284 to 347 of MDM2 for L11 interaction (59) and residues 216 to 302 for L23 binding (10, 24). We have recently determined that the MDM2 domain for L5 binding is located within residues 216 to 374 (data not shown). Thus, the three ribosomal proteins interact with MDM2 in an apparently overlapping but nonidentical fashion (Fig. 1B). Intriguingly, the small basic proteins that function to inhibit MDM2 and activate p53, including ARF, L5, L11, and L23, all interact with MDM2's conserved central acidic domain (Fig. 1B), suggesting that this area of MDM2 might serve as a site for receiving and integrating various MDM2-regulatory signals into the p53 pathway (8).

Previous studies have shown that cancer-associated alternative and aberrant splicing of *MDM2* mRNA occurs frequently. Many of the splicing variants retain the N-terminal p53 binding site and the C-terminal RING finger domain but lose the central acidic domain, including the zinc finger (3). Cancer-associated *MDM2* gene mutations are also reported. In a study involving 23 primary tumors of four types (osteosarcoma, non-Hodgkin's lymphoma, hepatocarcinoma, and leukemia), eight samples were found to contain mutations in the coding region

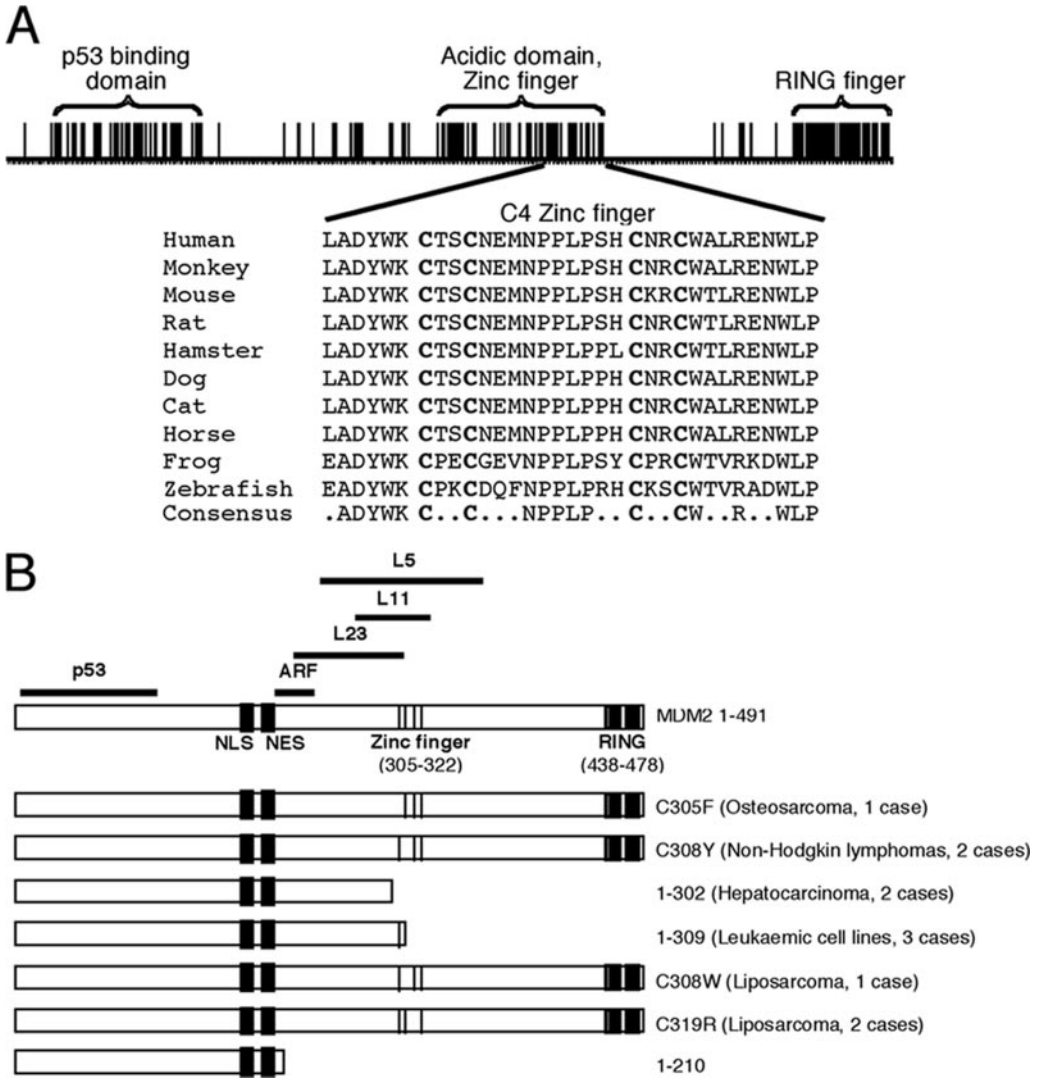

FIG. 1. Cancer-derived mutations target MDM2 C4 zinc finger. (A) Diagram of the three conserved MDM2 domains corresponding to an N-terminal p53-binding site, a central acidic region including the C4 zinc finger, and a C-terminal RING finger domain. Each vertical bar represents a residue conserved between the species shown. Sequence alignment of the MDM2 C4 zinc finger region from 10 species is shown. (B) Schematic representation of MDM2 structure. MDM2 mutations identified from human cancer (C305F, C308Y, positions 1 to 302 and 1 to 309) (47), C308W and C319R (51), and approximate binding areas for p53, ARF, and three ribosomal proteins are indicated.

of *MDM2* (47). Notably, most of these mutations target the central zinc finger of MDM2, including five cases of frameshift or nonsense mutations resulting in truncations of the zinc finger and three cases of missense mutations altering structurally important cysteine residues in the zinc finger (one affects C305 and two affect C308, as summarized in Fig. 1B). In a separate study involving 12 cases of liposarcoma (51), two were found to carry MDM2 mutations at the zinc finger (one at C308 and one at C319; Fig. 1B).

To determine if any functional consequences of MDM2 might result from these mutations, we constructed MDM2 mutants according to those identified in human cancer and examined the MDM2 protein complex by coupled [^{35}S]methionine metabolic labeling and immunoprecipitation (^{35}S-IP) using cell lysate from transfected U2OS cells. Comparison of wild-type and mutant MDM2 complexes revealed several cellular proteins with apparent molecular masses of 35, 32, and 20 kDa that associate specifically with the wild type but not with any of the mutant MDM2 proteins, and a 15-kDa protein associated with every mutant except MDM2$^{1\text{-}210}$, which contains a deletion from amino acid 211 to amino acid 491 (Fig. 2A). To determine the identity of these MDM2-associated proteins, a large-scale IP was carried out with extract from U2OS cells infected with adenovirus expressing the wild-type MDM2 (Ad-MDM2). Three of the four MDM2-binding polypeptides were identified by mass spectrometry as the ribosomal proteins L5, L11, and L23, corresponding to the 35-, 20-, and 15-kDa polypeptides, respectively (indicated in Fig. 2A), which have been described previously (4, 9, 24, 31, 35, 59). The identity of the 32-kDa peptide has not been established. To determine whether any of the cancer mutations might affect MDM2's interaction with p53 or ARF, p53 and ARF were

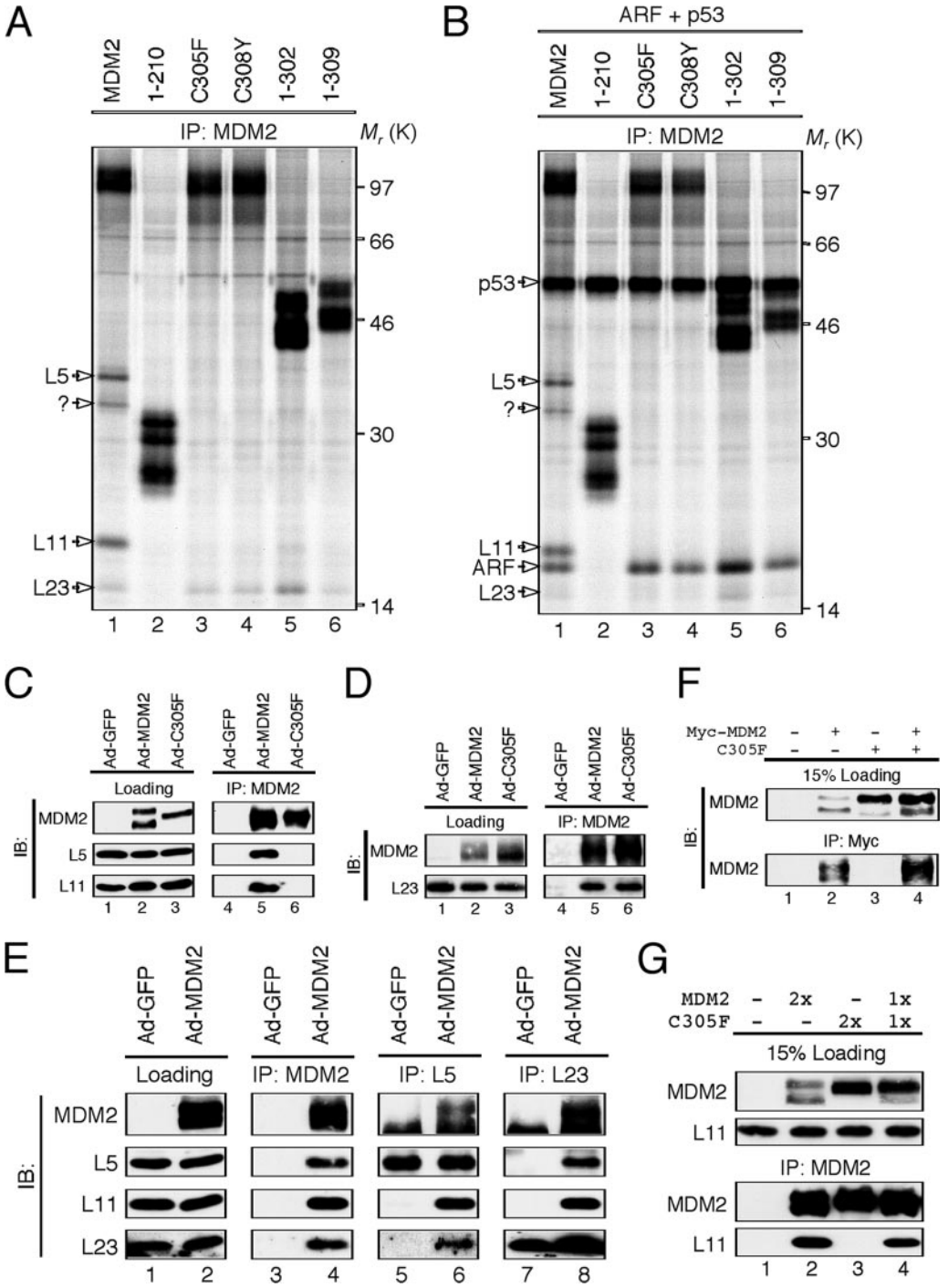

FIG. 2. The MDM2 zinc finger mutant fails to bind ribosomal proteins L5 and L11. (A and B) HeLa cells were transiently transfected with plasmid DNA expressing wild-type and mutant MDM2 as indicated. Twenty-four hours after transfection, cells were metabolically labeled with [^{35}S]methionine for 30 min, and cell lysates were immunoprecipitated with antibody to MDM2 (SMP14). Immunoprecipitated proteins were separated by SDS-PAGE gel and visualized by autoradiography. The molecular identities of L5, L11, and L23 were determined by protein mass spectrometry analysis following a preparative large-scale anti-MDM2 immunoprecipitation. (C and D) U2OS cells were infected with adenoviruses expressing either wild-type MDM2 or the MDM2^{C305F} mutant, and interactions of MDM2 with the endogenous ribosomal proteins were examined by IP-Western blotting. IB, immunoblotting. (E) L5, L11, and L23 do not immunoprecipitate each other but coexist in the same MDM2 immunocomplex. U2OS cells were infected with Ad-MDM2, and complex formation between MDM2 and endogenous L5, L11, and L23 was analyzed using antibodies recognizing each of these proteins. (F) A zinc finger mutant MDM2 is able to form homo-oligomers with wild-type MDM2. 2KO cells were transfected with Myc-tagged wild-type MDM2 and the untagged MDM2^{C305F} mutant as indicated, and IP-Western blotting was performed as indicated. (G) Expression of a zinc finger mutant does not interfere with the MDM2-L11 interaction. 2KO cells were transfected with MDM2 and mutant MDM2^{C305F} as indicated, followed by MDM2 immunoprecipitation and Western blotting for MDM2 and endogenous L11.

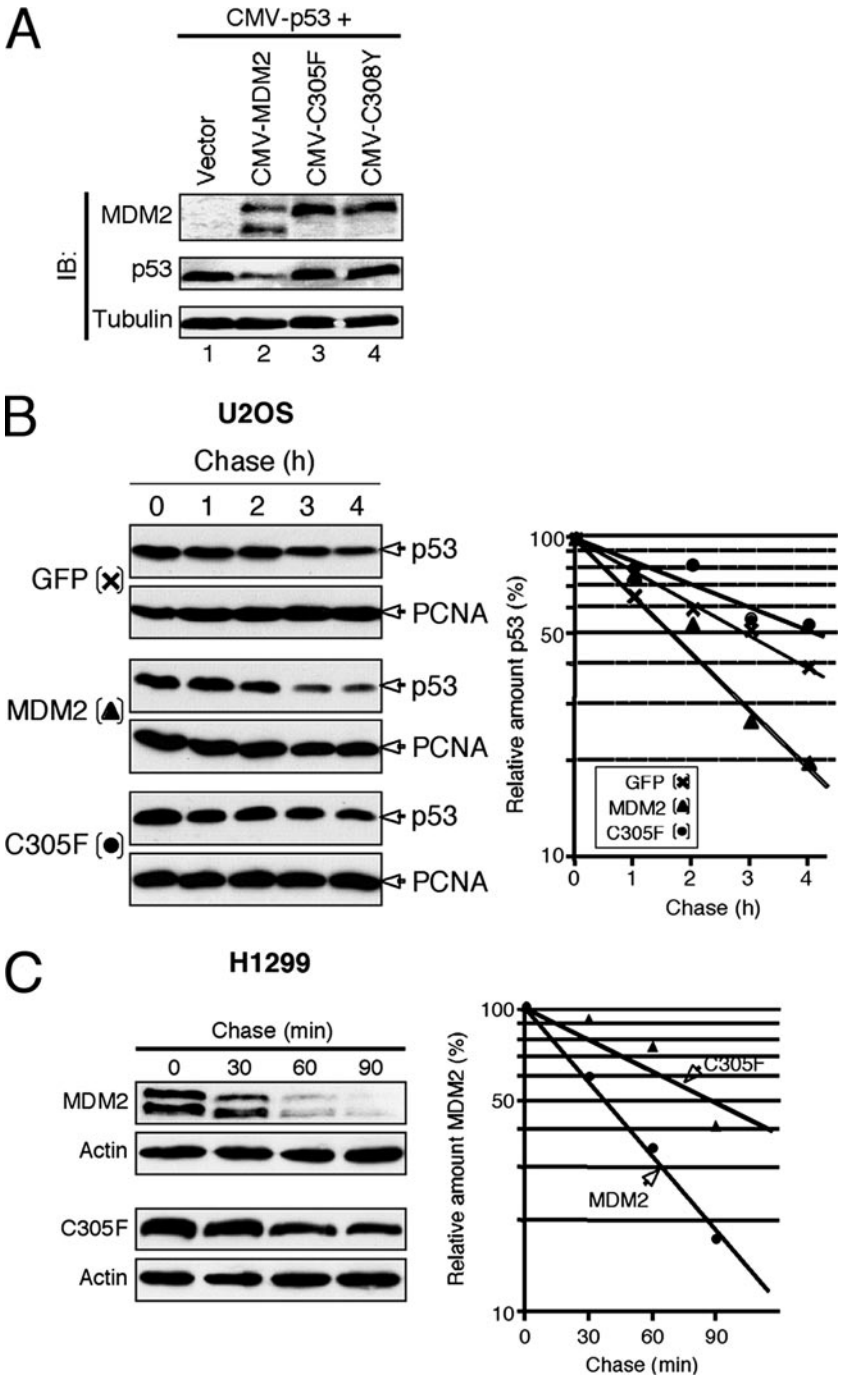

FIG. 3. MDM2 zinc finger mutants are attenuated in mediating p53 degradation. (A) U2OS cells were transfected with plasmids expressing p53 alone or in combination with MDM2, MDM2^{C305F}, or MDM2^{C308Y}, as indicated. The levels of p53, MDM2 and tubulin (loading control) were analyzed by Western blotting. IB, immunoblotting. (B) A p53 half-life assay in the presence of MDM2 or MDM2^{C305F} mutant. U2OS cells were infected with adenoviruses expressing wild-type MDM2, the MDM2^{C305F} mutant, or a GFP control. After 24 h of infection, de novo protein synthesis was blocked by addition of cycloheximide at 50 μg/ml, and the cells were chased for 0, 1, 2, 3, and 4 h, as indicated. Virus titers were adjusted so that cells expressed equal amounts of wild-type and mutant MDM2. Whole-cell lysates were prepared, and endogenous p53 and PCNA levels were analyzed by Western blotting using antibodies to p53 (DO1) and PCNA (PC10). Quantification of the results for p53 in each lane was normalized to the PCNA loading control, and the zero hour time point was set to 100%. (C) Half-life assays for MDM2 and MDM2^{C305F} mutant. H1299 cells (p53-negative, MDM2 undetectable) were transfected with plasmids encoding wild-type or mutant MDM2^{C305F}. Cycloheximide (50 μg/ml) was added to the cells 24 h after transfection. The cells were lysed at indicated time points, and the level of MDM2 was determined by Western blotting. The percentage of MDM2 remaining at each time point was plotted to the right.

coexpressed with each MDM2 mutant and the MDM2 protein complexes were examined. As shown in Fig. 2B, none of the cancer-derived mutations affected MDM2's association with either p53 or ARF. As expected, the control deletion mutant, $MDM2^{1-210}$, retained binding activity toward p53 but not ARF (Fig. 2B, lane 2). Hence, our results indicate that mutations on or near the MDM2 central zinc finger disrupt its association with L5 and L11, but not L23 or ARF.

To further study the function of the MDM2 zinc finger, we generated an adenovirus expressing the C305F mutant MDM2 (Ad-C305F). We expressed the mutant and wild-type MDM2 in U2OS cells and examined the interaction of each with endogenous ribosomal proteins by IP-Western blotting. Consistent with the results obtained from ^{35}S-IP, $MDM2^{C305F}$ did not interact with L5 or L11 but retained the ability to interact with L23 (Fig. 2C and D). Notably, the $MDM2^{C305F}$ protein migrated as a single band, as opposed to the wild-type MDM2's two bands, on the SDS-polyacrylamide gel electrophoresis (PAGE) gel (Fig. 2C, lane 3). The change in MDM2's banding pattern was most obvious in the direct Western blot, while less distinct following an immunoprecipitation. The nature of this banding pattern change remains to be determined. To examine whether the three ribosomal proteins can either coexist together with MDM2 or compete for MDM2 binding, we infected U2OS cells with Ad-MDM2 and examined complex formation between MDM2 and endogenous L5, L11, and L23 by antibodies raised against each of the ribosomal proteins. We found that L5, L11, and L23, although found together in the large ribosomal subunit, did not interact directly with each other in the immunoprecipitation, but could coexist together with MDM2 in an MDM2 immunocomplex (Fig. 2E). These results are consistent with a previous study (9). Because MDM2 can form homo-oligomers through the interaction of its C-terminal RING domain (52), we wanted to test whether the zinc finger mutation might affect MDM2 homo-oligomerization. Using a Myc-tagged wild-type MDM2 and a nontagged mutant, we determined that the zinc finger mutation did not affect MDM2's ability to form homo-oligomers (Fig. 2F). We then analyzed whether expression of the $MDM2^{C305F}$ mutant interferes with the wild-type MDM2-L11 interaction. To accomplish this, MDM2 and the $MDM2^{C305F}$ mutant were expressed to equal levels either individually or in a 1:1 combination in $MDM2^{-/-}$; $p53^{-/-}$ (2KO) MEF cells, and the interaction of MDM2 with endogenous L11 was determined by IP-Western blotting. We chose to use 2KO cells to avoid endogenous MDM2 interference. As shown in Fig. 2G, the level of L11 that was precipitated from cells expressing a 1:1 combination of MDM2 and $MDM2^{C305F}$ was approximately half of that pulled down from cells expressing 2× MDM2, indicating that expression of the $MDM2^{C305F}$ mutant does not inhibit the interaction of L11 with wild-type MDM2. Together, these data indicate that the zinc finger mutation selectively disrupts MDM2's interaction with L5 and L11, but not L23, and does not affect MDM2 homo-oligomerization.

The $MDM2^{C305F}$ mutant is attenuated in mediating p53 degradation. To further study the functional consequences of the MDM2 zinc finger mutation, we examined MDM2 zinc finger mutants in promoting p53 degradation. U2OS cells were transfected with plasmids expressing p53 alone or in combination with MDM2, $MDM2^{C305F}$, or $MDM2^{C308Y}$ (with both MDM2 mutants constructed according to mutations identified in human cancer) (47), and the levels of p53 were determined by Western blotting. Under conditions in which overexpression of wild-type MDM2 resulted in p53 degradation, overexpression of the zinc finger mutant MDM2 had no discernible effect on p53 protein level (Fig. 3A). This attenuated ability of MDM2 zinc finger mutants to induce p53 degradation was further demonstrated by a half-life assay of endogenous p53 under the control of ectopically expressed MDM2. In this assay, U2OS cells (p53 positive) were infected with adenoviruses expressing GFP, MDM2, or $MDM2^{C305F}$. Twenty-four

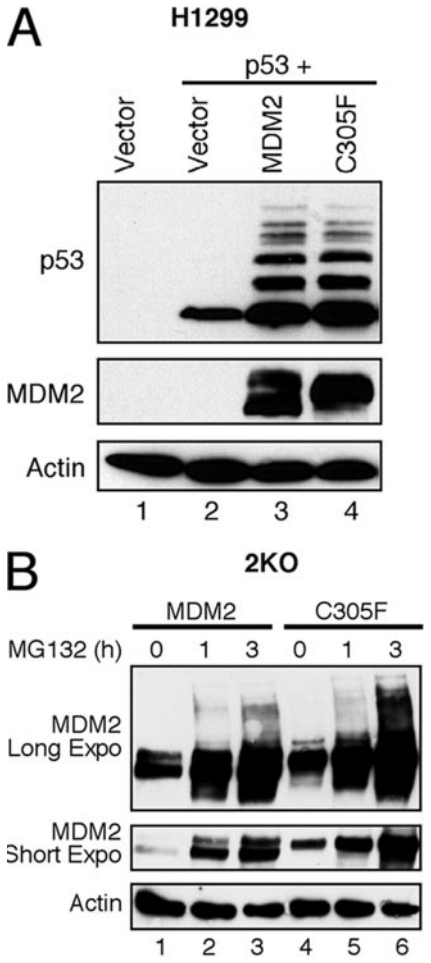

FIG. 4. The $MDM2^{C305F}$ mutant retains the ability to promote ubiquitination of p53 and itself. (A) MDM2 and $MDM2^{C305F}$ mutant promote p53 ubiquitination. H1299 cells were transfected with plasmids expressing p53, wild-type MDM2, or the $MDM2^{C305F}$ mutant as indicated. The cells were treated with 10 μM MG132 4 h before harvesting to allow accumulation of ubiquitinated p53. Cells were then lysed in hot SDS lysis buffer, and the expression of p53 and MDM2 was analyzed using Western blotting with antibodies to p53 (DO1) or MDM2 (4B11). Both wild-type MDM2 and $MDM2^{C305F}$ promoted p53 ubiquitination, as evidenced by p53 Ub-ladder formation. (B) MDM2 and $MDM2^{C305F}$ mutant promote self-ubiquitination. 2KO cells were transfected with plasmids encoding wild-type MDM2 or the $MDM2^{C305F}$ mutant and incubated with MG132 (10 μM) for the indicated times. Expression of MDM2 was detected by an MDM2 antibody (4B11). Both long and short exposures (Expo) of the MDM2 blot are shown.

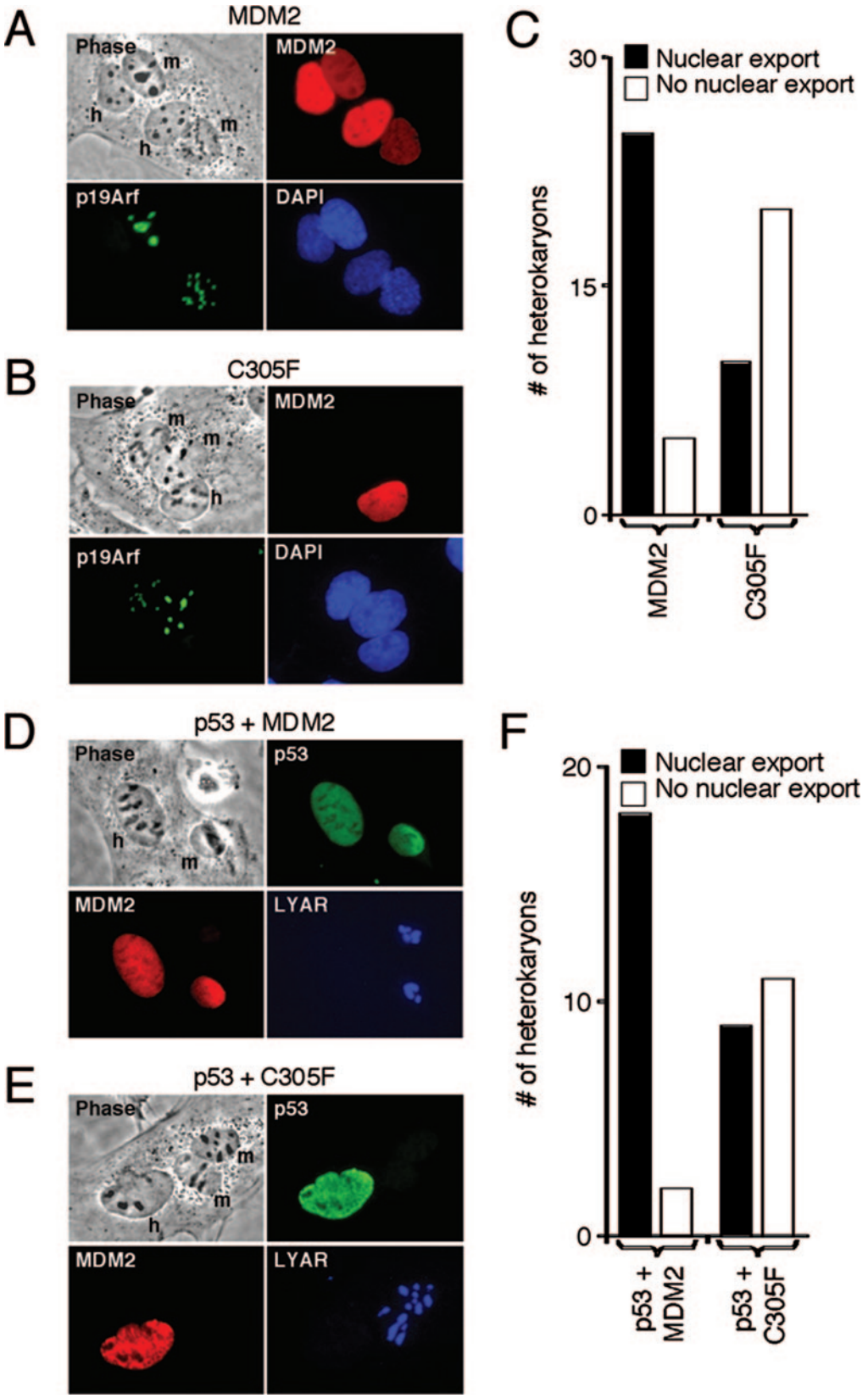

FIG. 5. MDM2^{C305F} mutant is impaired in undergoing nuclear export. (A and B) MDM2 nuclear export determined by a heterokaryon assay. Saos2 cells were transiently transfected with plasmids expressing either wild-type MDM2 or the MDM2^{C305F} mutant. One day after transfection, the cells were fused with 2KO MEF cells by polyethylene glycol in the presence of cycloheximide (50 μg/ml, to block de novo protein synthesis). MDM2 (red) and endogenous p19Arf (green) were detected by a mouse anti-MDM2 antibody and a rabbit anti-p19Arf antibody, respectively. Formation of

hours after infection, the cells were treated with cycloheximide. The levels of endogenous p53 were then determined by Western blotting at different time intervals, and the results were quantified and plotted (Fig. 3B). Without MDM2 overexpression, ectopically expressed p53 had a half-life of approximately 200 min in U2OS cells (Fig. 3B, top panel). Coexpression of MDM2 reduced the half-life of p53 to approximately 100 min (middle panel). Coexpression of the MDM2^{C305F} mutant, in contrast, did not reduce p53's half-life (bottom panel). The attenuated p53 degradation by the mutant MDM2 was further confirmed with ^{35}S-metabolic labeling in an independent experiment and in other cell lines, including 2KO (data not shown). Thus, whereas wild-type MDM2 promotes p53 degradation, the MDM2^{C305F} mutant is impaired in doing so. We also examined the effect of the zinc finger mutation on the protein stability of MDM2 itself. We used H1299 cells (p53 negative, MDM2 undetectable) for the experiment to circumvent potential effects from endogenous p53. Interestingly, we found that while the wild-type MDM2 had an estimated half-life of about 40 min, the mutant had a significantly longer half-life of about 90 min (Fig. 3C), indicating that the mutant protein is more stable than the wild type. In summary, these experiments find that the MDM2 zinc finger is critical in mediating MDM2-induced p53 degradation as well as MDM2 self-degradation.

The MDM2^{C305F} mutant can promote p53 ubiquitination. Both p53 and MDM2 are degraded through ubiquitin-mediated proteolysis, and MDM2 functions as the E3 ligase for both. To determine whether the attenuated p53 degradation is due to an inability of the MDM2^{C305F} mutant to promote p53 ubiquitination, we carried out an in vivo p53 ubiquitination assay. H1299 cells were transfected with plasmids expressing MDM2 and p53. The cells were treated with the proteasome inhibitor MG132 prior to lysis to allow ubiquitinated proteins to accumulate. Subsequently, the cells were lysed in hot SDS lysis solution (hot SDS solution protects ubiquitinated protein species from deubiquitination), and the p53 protein was detected directly by straight Western blotting with an anti-p53 antibody. To our surprise, the MDM2^{C305F} mutant, although attenuated in promoting p53 degradation, was as active as the wild-type MDM2 in promoting p53 ubiquitination (Fig. 4A). The levels of p53 ubiquitination were essentially equal in samples transfected with MDM2 or the MDM2^{C305F} mutant, indicating that the zinc finger mutation did not affect MDM2's E3 ubiquitin ligase activity toward p53. The ability of the MDM2^{C305F} mutant to ubiquitinate p53 was further confirmed by an IP-Western blot assay of polyubiquitinated p53 in another experiment (see below in Fig. 6). To gain further insight into the E3 ubiquitin ligase function of the MDM2^{C305F} mutant, we examined self-ubiquitination of the mutant MDM2 in 2KO cells. As shown in Fig. 4B, in the absence of endogenous p53 and Mdm2, the MDM2^{C305F} mutant was fully capable of promoting self-ubiquitination. We have noticed that treatment of the cells with MG132 dramatically stabilized wild-type MDM2 (Fig. 4B, compare lane 1 with lane 2 in the short exposure), but less so for the MDM2^{C305F} mutant (Fig. 4B, compare lane 4 with lane 5 in the short exposure), which is in agreement with the observed longer half-life of the MDM2^{C305F} mutant than the wild type MDM2 (Fig. 3C). Hence, our data show that the integrity of the zinc finger is essential for MDM2 to efficiently induce degradation of p53 and itself, but is not strictly required for ubiquitination of either one.

The MDM2^{C305F} mutant is impaired in nuclear export. It has been shown that MDM2-mediated p53 ubiquitination occurs in both the nucleus (16) and the cytoplasm (40) and that MDM2 nucleocytoplasmic shuttling contributes to p53 degradation (46). The uncoupling of normal p53 ubiquitination with an attenuated degradation by the MDM2^{C305F} mutant prompted us to examine whether the mutant MDM2 was unable to undergo nucleocytoplasmic shuttling, thus trapping p53 in the nucleus. For this purpose, we carried out an interspecies heterokaryon assay to examine directly the nucleocytoplasmic shuttling of mutant MDM2 and p53. Human Saos2 (MDM2 undetectable) cells were transiently transfected with plasmids expressing either wild-type MDM2 or the MDM2^{C305F} mutant. One day after transfection, the cells were fused with 2KO MEF cells by polyethylene glycol in the presence of cycloheximide (50 μg/ml, to block de novo protein synthesis). 2KO cells express high levels of p19Arf that can be used to distinguish donor human nuclei from recipient mouse nuclei in heterokaryons. Accumulation of the MDM2 protein in the mouse nuclei in a heterokaryon will indicate that the protein has been exported out of the human nucleus into the shared cytoplasm and then imported into the mouse nucleus. Using this technique, we detected relocalization of wild-type MDM2 to the mouse nucleus in a significant portion of heterokaryons (Fig. 5A and C). In contrast, in the majority of heterokaryons expressing the MDM2^{C305F} mutant, the protein did not enter the mouse nucleus (Fig. 5B and C). Thus, the MDM2^{C305F} mutant is deficient in nuclear export. To determine whether the nuclearly trapped MDM2^{C305F} mutant might affect p53 nuclear export, we expressed wild-type MDM2 or the MDM2^{C305F} mutant together with p53 in a heterokaryon assay. p53 (green) and MDM2 (red) were detected by rabbit anti-p53 and mouse anti-MDM2 antibodies, respectively, and the mouse nuclei were identified by a rat anti-LYAR antibody, which specifically detects mouse nucleoli with high sensitivity (50). In samples expressing wild-type MDM2, p53 was readily detected in most mouse nuclei in the heterokaryons (Fig. 5D

heterokaryons is shown in phase-contrast images, with human (h) and mouse (m) nuclei indicated. Relocalization of MDM2 to the mouse nucleus was seen in a significant portion of heterokaryons expressing wild-type MDM2 but not mutant MDM2, as shown with quantification in panel C. DAPI, 4′,6′-diamidino-2-phenylindole. (D and E) MDM2 and p53 nuclear export determined by a heterokaryon assay. Saos2 cells were transfected with plasmids expressing wild-type MDM2 or the MDM2^{C305F} mutant together with a p53-expressing plasmid. The fusion was performed as described above. MDM2 (red) and p53 (green) were detected by mouse anti-MDM2 and rabbit anti-p53 antibodies, respectively. The mouse nuclei were detected by a rat anti-LYAR (blue) antibody. Formation of heterokaryons is shown in phase-contrast images, with human (h) and mouse (m) nuclei indicated. Relocalization of MDM2 and p53 to the mouse nucleus was seen in about half of the heterokaryons examined, as shown with quantification in panel F.

and F). In contrast, in samples expressing the MDM2^{C305F} mutant, a significantly higher proportion of heterokaryons showed retention of p53 in the Saos2 nucleus (Fig. 5E and F), suggesting that the nuclear export-defective MDM2^{C305F} mutant trapped p53 in the nucleus. Thus, the impaired nuclear export of p53 by coexpression of the MDM2^{C305F} mutant is, at least in part, an explanation for the defect in p53 degradation.

The MDM2^{C305F} mutant retains the function of repressing p53 transcriptional activity. Because MDM2 is able to repress the transcriptional activity of p53 through binding to and masking the p53 N-terminal transactivation domain (42, 54), we wanted to determine whether the MDM2^{C305F} mutant retains p53-suppressive activity. To this end, U2OS cells were coinfected with p53 together with Ad-MDM2 or Ad-MDM2^{C305F}, and the levels of endogenous p21 were examined by Western blotting. Figure 6A showed that p21 was activated by p53, and this activation was eliminated by coexpression of either MDM2 or MDM2^{C305F}, indicating that the MDM2^{C305F} mutant retained the capability of suppressing p53. To determine whether the MDM2^{C305F} mutant was equally effective as the wild-type MDM2 in repressing p53, we adjusted the ratio of p53 to MDM2 in the transfections so that the p53 protein was not degraded and its activity was partially suppressed. Intriguingly, we found that the MDM2^{C305F} mutant appeared to be more effective than the wild-type protein in suppressing p53 (Fig. 6B, lane 3). To study the zinc finger mutant MDM2-imposed p53 suppression in further detail, we examined p53 transcriptional activity using a p53-responsive reporter construct, pGL13-Luc (11), in a luciferase assay (Fig. 6C). Cotransfection of pGL13-Luc with a p53-expressing plasmid led to an average six- to sevenfold increase in luciferase activity, which was normalized as 100% of p53-dependent transactivation. Coexpression of p53 with either wild-type MDM2 or MDM2^{C305F} decreased the luciferase activity in a dose-dependent manner. We noticed that, again, the MDM2^{C305F} mutant consistently exhibited stronger inhibition than did the wild-type MDM2 at each dosage tested. We speculate that the higher p53-inhibitory activity of the MDM2^{C305F} mutant might be related to its nuclear retention and longer half-life. Together, these results indicate that mutations in the zinc finger motif, while impairing MDM2-mediated p53 degradation, do not reduce and may actually enhance, MDM2's ability to repress p53's transactivation activity, suggesting a potential explanation for the presence of MDM2 zinc finger mutations in human cancers.

The MDM2^{C305F} mutant escapes from L11 inhibition. Previous studies have shown that the interaction of ribosomal protein L11 with MDM2 inhibits MDM2's p53-suppressive function (31, 59) and plays a critical role in mediating growth inhibition-induced p53 activation (4). To determine whether the MDM2^{C305F} mutant, by breaking off L11 binding, might have escaped L11-imposed inhibition, we examined the effect of L11 overexpression on MDM2-mediated p53 repression using a luciferase assay. As shown in Fig. 7A, under conditions in which wild-type MDM2 effectively repressed p53-dependent transactivation of the pGL13-Luc reporter (column 4), coexpression of L11 restored up to 70% of p53 activity (column 5). In contrast, L11 did not relieve MDM2^{C305F}-imposed p53 repression (columns 6 and 7), indicating that MDM2^{C305F} is insensitive to L11 inhibition, supporting the hypothesis that the mutant MDM2 escapes from L11 inhibition.

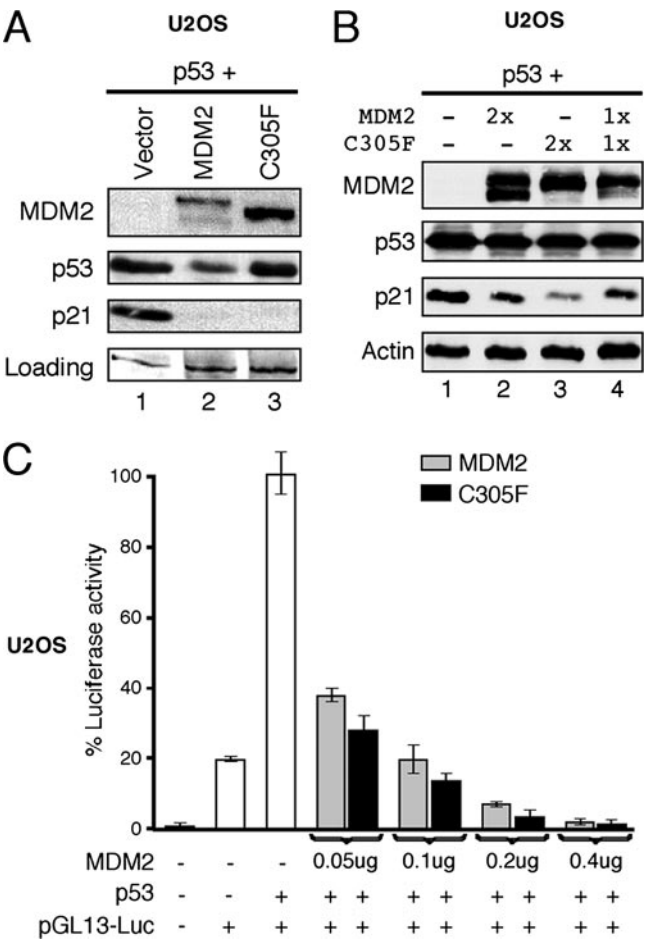

FIG. 6. The MDM2^{C305F} mutant retains the ability to repress p53 transcriptional activity. (A) Suppression of p53-induced endogenous p21 by MDM2. U2OS cells were infected with adenoviruses expressing MDM2 or MDM2^{C305F}. Twenty-four hours after infection, cell lysates were prepared and separated by SDS-PAGE. Different portions of the same blot were immunoblotted with antibodies recognizing MDM2, p53, and p21 as indicated. A nonspecific protein detected by the MDM2 antibody serves as a loading control. (B) Apparent higher p53-suppressive activity of MDM2^{C305F} mutant. U2OS cells were transfected with equal amounts of total DNA expressing p53 and MDM2 (or MDM2^{C305F}) in a 1:1 ratio. This ratio of p53 and MDM2 plasmid transfection does not trigger p53 degradation, making a comparative analysis of p21 suppression possible. Levels of MDM2, p53, and endogenous p21 were determined by Western blotting. (C) Suppression of p53 transcriptional activity by MDM2 in a luciferase assay. U2OS cells were cotransfected with a p53-responsive pGL13-Luc reporter plasmid along with plasmid expressing the indicated proteins. Twenty-four hours after transfection, clarified cell lysates prepared from each transfected cell population were incubated with a luciferase assay buffer, and optical density at 595 nm was determined with a luminometer. The luciferase activity for each sample was normalized to β-galactosidase activity for transfection efficiency.

To gain further insight into the mechanism of the L11-imposed MDM2 inhibition that is abrogated by the zinc finger mutation, we carried out an in vivo p53 ubiquitination assay by transfecting U2OS cells with plasmid DNA expressing HA-Ub together with p53, MDM2, ARF, and L11 in a variety of combinations. One day after transfection, cells were lysed in hot SDS lysis solution, and p53 was pulled down with an anti-

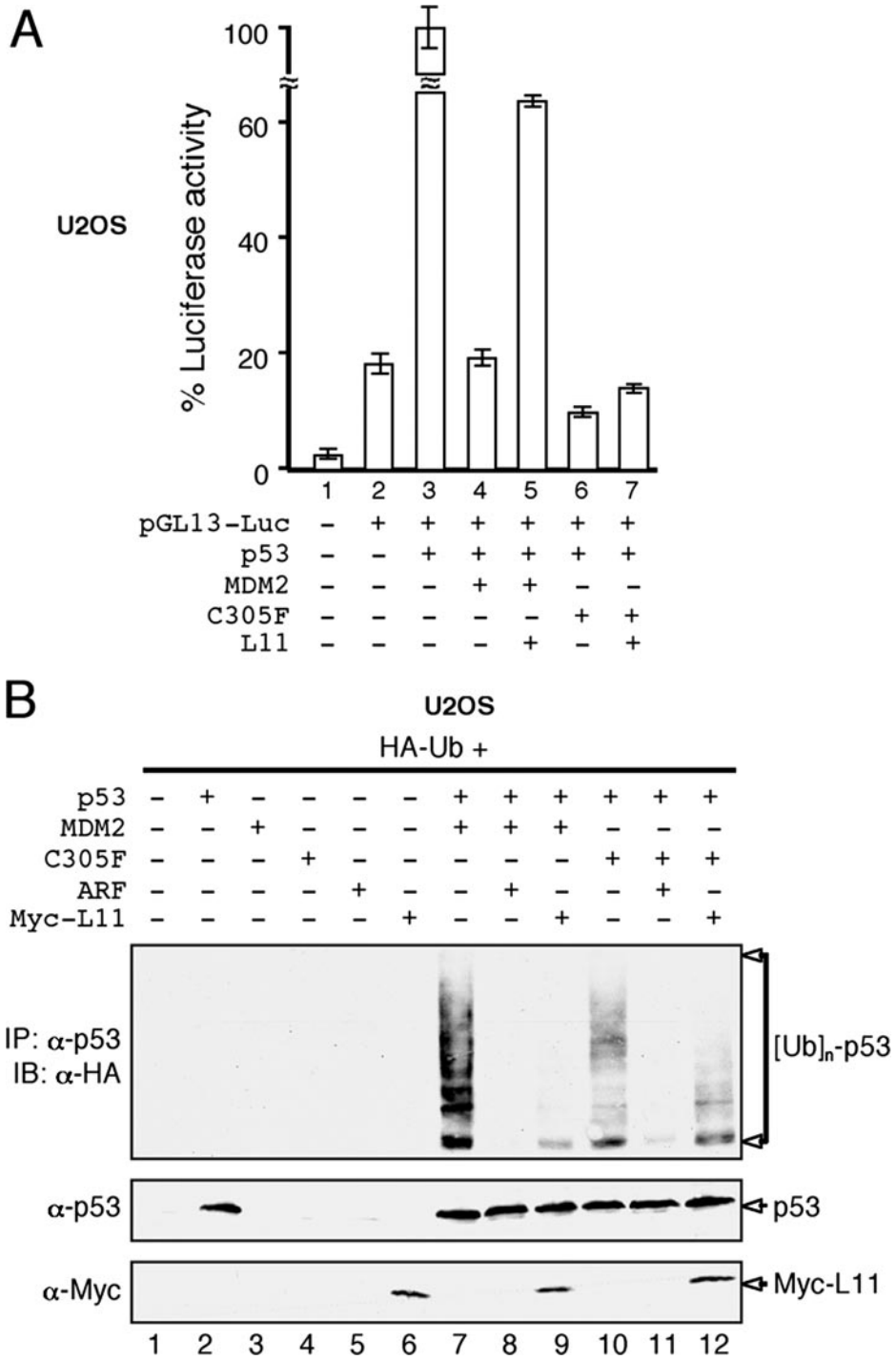

FIG. 7. The MDM2^{C305F} mutant escapes negative regulation from ribosomal protein L11. (A) L11 reverses MDM2-suppressed p53 transcriptional activity in a luciferase assay. pGL13-Luc reporter plasmid was cotransfected with plasmids expressing the indicated proteins in U2OS cells. The luciferase assay was carried out as described above. (B) The MDM2^{C305F} mutant escapes inhibition of L11 but not ARF in a p53 ubiquitination assay. U2OS cells were transfected with plasmids in various combinations as indicated. Twenty hours after transfection, cells were treated with proteasome inhibitor MG132 (50 μM) for 4 h prior to lysis. Clarified cell lysate was immunoprecipitated with anti-p53 (α-p53) antibody (FL393), and the immunoprecipitates were resolved by SDS-PAGE followed by immunoblotting (IB) with anti-HA (α-HA) antibody. α-Myc, anti-Myc antibody.

p53 antibody and separated on an SDS-PAGE gel. HA-Ub-conjugated p53 was then detected with an anti-HA antibody. As shown in Fig. 7B, polyubiquitinated p53 was detected in abundance when MDM2 was cotransfected (lane 7). The ubiquitinated p53 was not detected when ARF or L11, both of which inhibit the ubiquitin ligase activity of MDM2, were included in the transfection (lanes 8 and 9). Conversely, in agreement with previous data showing that MDM2^{C305F} is capable

of promoting p53 monoubiquitination (Fig. 4A), a smaller but still considerable amount of polyubiquitinated p53 was detected when MDM2^{C305F} was cotransfected (lane 10). Strikingly, however, the MDM2^{C305F} mutant-induced p53 ubiquitination was inhibited effectively by ARF (lane 11) but only partially by L11 (lane 12), indicating that the mutant MDM2^{C305F} escapes specifically from L11- but not ARF-imposed inhibition.

DISCUSSION

Sequence alignment has revealed three highly conserved areas in the MDM2 protein, located at the N terminus, the C terminus and the central acidic domain (Fig. 1A). The N-terminal conserved domain is essential for p53 binding, whereas the C terminus possesses a RING finger domain responsible for MDM2's E3 ubiquitin ligase activity. However, the function of the central acidic domain, which includes a highly conserved C4 zinc finger, has been elusive. MDM2 interacts with multiple ribosomal proteins, including, but not necessarily limited to, L5, L11, and L23. Although there are variations in published reports regarding the exact interacting sequences on MDM2 for each of the three ribosomal proteins, all point to the central acidic domain (Fig. 1B), indicating that this area of MDM2 could have a function that is modulated by ribosomal protein interaction. A potential biological role for the MDM2 zinc finger was suggested by the identification of human cancer-derived mutations that specifically target the cysteine residues in MDM2's zinc finger motif (47, 51). In our present study, we examined two of these cancer-associated MDM2 mutations and have identified an essential role for the zinc finger of MDM2. We show that a mutation at a zinc-coordinating cysteine residue specifically disrupts MDM2 binding with ribosomal proteins L5 and L11, but not L23 or ARF, and the mutant MDM2 remains fully functional in binding with and suppressing the activity of p53. It has been shown previously that the interaction of ribosomal proteins with MDM2 mediates signals of ribosomal stress and growth inhibition to induce a p53-dependent cell cycle arrest (4). This regulation of p53 is thought to occur through the binding of ribosomal proteins to the central acidic domain of MDM2, thereby inhibiting MDM2's E3 ubiquitin ligase function and consequently stabilizing and activating p53 (9, 24, 31, 59). Thus, MDM2 with mutations disrupting ribosomal protein binding would evade ribosomal stress-induced growth inhibition, which could explain the origin of these zinc finger mutations in human cancer.

Why the zinc finger is critical for MDM2 to interact with L5 and L11 is unclear, but it is likely that a correct structure formed by the C4 zinc finger is a minimal requirement for the binding. A recently solved solution structure of the MDM2 C4 zinc finger shows that residues 297 to 329 (numbers based on human MDM2 sequence) form a compact globular fold in which a zinc ion is coordinately bound by the four cysteine residues (C305, C308, C319, and C322) (58). The MDM2 C4 zinc finger is a member of the RanBP2/NZF-like zinc finger family, which also includes functionally diverse proteins such as RanBP2, Npl4, Vps36p, Znf265, and EWS (38). The C4 zinc finger structure in RanBp2/NZF proteins has been implicated in RNA binding and direct interaction with ubiquitin (1, 38). However, the MDM2 C4 zinc finger failed to interact with the ubiquitin molecule in vitro (38). It is likely that the C305F mutation in MDM2 leads to a collapse of the zinc finger fold (58). Our results showing that the C4 zinc finger integrity is required for binding with ribosomal proteins L11 and L5 is consistent with this notion (Fig. 2). With the availability of the MDM2 C4 zinc finger solution structure, it is possible to use additional site-directed mutagenesis of nonstructural zinc finger amino acids to compare the roles of the MDM2 and MDMX C4 zinc fingers, which would be of particular interest given the lack of conservation between MDM2 and MDMX in some of the nonstructural amino acids in the zinc finger domain.

We have found that the MDM2^{C305F} mutant, when expressed ectopically, has a delayed protein turnover (Fig. 3C). Although both MDM2 and MDM2^{C305F} are relatively short-lived, the mutant has a half-life twice as long as that of wild-type MDM2 (90 min versus 40 min). This can be attributed, at least in part, to the attenuated ability of the MDM2^{C305F} mutant to undergo nuclear export (Fig. 5). How the zinc finger mutation, which is quite far from MDM2's NES sequence (see Fig. 1B), attenuates MDM2 nuclear export is unknown. The observation that the mutation disrupts the L5-L11 interaction brings about an interesting possibility: L5 and L11 may help MDM2 to undergo nuclear export. This idea may not be surprising, given that ribosomal proteins are known to travel back and forth between the nucleolus and cytoplasm and that L5 has been shown to be involved in shuttling of rRNA out of the nucleus (17). On the other hand, because we have found that a zinc finger mutation significantly affects the MDM2 conformation such that it migrates as a single band rather than a doublet on the SDS-PAGE gel (Fig. 2C), we cannot exclude that conformational changes in MDM2 might affect its nuclear export. A long-lived, nuclearly trapped MDM2 may gain an advantage in binding to and suppressing the function of p53. This notion is in line with the observation that the zinc finger mutant MDM2 was originally found in human cancers and is reported to accumulate to high levels in these tumors (43, 51).

It has been shown previously that deletion of residues 222 to 272 attenuates MDM2-mediated degradation, but not ubiquitination, of both MDM2 itself and p53 (2), indicating a mechanism that could disintegrate MDM2-induced p53 ubiquitination from degradation. We have found that a single mutation in the zinc finger did not affect MDM2-induced p53 ubiquitination appreciably, but rather attenuated MDM2-induced p53 degradation (Fig. 3 and 4). A possible explanation for the apparently unchanged ubiquitination and the slower turnover of p53 in the presence of the MDM2 mutant could be that the mutant MDM2, although able to ubiquitinate p53, is itself impaired in nuclear export (Fig. 5) and thus traps p53 in the nucleus and attenuates its degradation.

It is conceivable that the ribosomal protein-MDM2-p53 connection represents a signaling pathway that functions to safeguard the integrity of ribosomal biogenesis in higher-order eukaryotic cells in order to coordinate cellular growth with proliferation. In this regard, mutations in genes that affect the function of this pathway could arise in human cancers, granting an advantage to cells in escaping p53 surveillance. MDM2 gene amplification is a predominant mechanism described for MDM2 oncogenic activation and has been detected in many types of human cancers, including soft tissue sarcomas (41) and

brain tumors (7, 45). Thus far, most studies examining MDM2 oncogenic alterations have focused on analyzing MDM2 gene amplification and alternative splicing (3). Our findings that tumor-derived mutations targeting the central zinc finger disrupt the negative regulation of MDM2 by L11 represent a novel putative mechanism for MDM2 oncogenic activation. Five different types of tumors represented by these limited studies contain missense or nonsense mutations in MDM2 that disrupt its interaction with L5 and L11. The possibility formally exists that the prevalence of gene mutation-based MDM2 oncogenic activation may be underestimated. Based on the current and previous studies, it is tempting to propose that the MDM2-p53 feedback loop has evolved to safeguard cells from undergoing uncontrolled growth and proliferation through three major signaling pathways: the phosphorylation of MDM2 and p53 by a variety of kinases signals DNA damage, the ARF-MDM2 interaction signals oncogenic insults, and the ribosomal protein-MDM2 interaction signals malfunctions in ribosomal biogenesis.

ACKNOWLEDGMENTS

We thank Koji Itahana, Hilary Clegg, and Theresa Allio for their helpful advice and technical assistance.

M.L. was supported by a postdoctoral fellowship from the Swedish Research Council. This study was supported by grants from the NIH, the Burroughs Wellcome Fundation, and the Leukemia Research Foundation to Y.Z.

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