

Hdmx Recruitment into the Nucleus by Hdm2 Is Essential for Its Ability to Regulate p53 Stability and Transactivation*

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The Hdmx gene product is related to the Hdm2 oncoprotein, both of which interact with and regulate p53 stability and function. Like Hdm2, Hdmx is able to inhibit p53 transactivation; however, at variance with Hdm2, which promotes ubiquitination, nuclear export, and degradation of p53, Hdmx increases p53 stability. We report here (i) that overexpressed Hdmx is cytoplasmic and Hdm2 recruits Hdmx into the nucleus and (ii) that nuclear Hdmx blocks Hdm2-mediated nuclear export of p53 and down-regulates p53-dependent transcription. Furthermore we showed that Hdmx inhibits Hdm2-mediated p53 ubiquitination. It appears, therefore, that a regulatory loop exists in which Hdm2 regulates the intracellular localization of Hdmx, and nuclear Hdmx regulates several functions of Hdm2 (ubiquitin ligase activity and p53 nuclear export).

p53 is the most frequently inactivated tumor suppressor gene in human cancer irrespective of the tumor type, site, and patient age. In cells undergoing DNA damage or responding to certain forms of stress (replicative senescence, hypoxia, oxidative stress, and oncogene overexpression) the ordinarily short-lived p53 protein is stabilized, rapidly accumulates, and undergoes several activating post-translational modifications. In this context, activated p53, via its ability to regulate gene transcription, elicits either a cell cycle arrest or apoptosis (1). As it would be expected, p53 actions are largely regulated by post-translational events, including direct binding and occlusion of the transactivation domain, ubiquitination and degradation by the proteasome, and control of subcellular localization. The Hdm2 protein has been implicated in all three of these processes, most recently as mediator of the export of p53 from the nucleus, which enhances the degradation of p53 in cytoplasmic proteasomes (2–5). Hdm2 functions as an E3¹ ubiquitin ligase for p53, and the C-terminal RING-finger domain of Hdm2 is critical for ligase activity (6, 7). Interestingly the *Hdm2* gene itself is a transcriptional target of p53. Thus, there is evidence for an autoregulatory feedback loop involving the expression and

function of Hdm2 and p53. From a genetic standpoint, the close relationship between the murine ortholog of Hdm2, Mdm2, and p53 is further supported by the striking observation that a p53-null state can completely rescue the early-embryonic-lethal phenotype associated with Mdm2 deficiency (8, 9).

Hdmx is structurally related to Hdm2, and a significant similarity is especially notable in the p53-binding domain and in the C-terminal RING-finger domain (10). Hdmx has been shown to bind p53 and inhibit p53 transactivation in specific cellular contexts but, unlike Hdm2, is unable to induce p53 degradation (10, 11). Similar conclusions were drawn when using the Hdmx murine ortholog Mdmx (12, 13). Moreover, Mdmx and Mdm2 form stable heterodimers through their C-terminal RING-finger domains, and this interaction results in a substantial increase in the steady-state levels of Mdm2 (14, 15). Finally it has been reported that both Mdmx (13) and Hdmx (11), through a yet unknown mechanism, are able to interfere with Mdm2-mediated p53 degradation, resulting in an increase in p53 protein levels while maintaining suppression of p53 transactivation. It was therefore proposed that Hdmx (and Mdmx) secures a nuclear pool of transcriptionally incompetent p53, which upon stress can be quickly activated. Finally the first genetic evidences confirming the role of Mdmx as a critical regulator of p53 function *in vivo* are now available. Mdmx-mutant mice die around midgestation because of loss of cell proliferation, while loss of p53 completely rescues this embryonic lethality (16).² These new exciting data raise the possibility that increased Mdmx levels and the resulting inhibition of p53 transcriptional activity contribute to development of human tumors.

We show here that exogenous Hdmx is mainly localized in the cytoplasm of transfected cells by indirect immunofluorescence, while when co-expressed with Hdm2, Hdmx was then found almost exclusively in the nucleus. We show that this effect is dependent on the integrity of the RING-finger domain of both Hdmx and Hdm2 proteins. Furthermore, we demonstrate that the Hdm2-dependent nuclear recruitment of Hdmx is indispensable for its ability to exert its two previously reported activities, regulation of p53 steady state levels and block of p53 transcriptional ability.

EXPERIMENTAL PROCEDURES

Plasmids—DNAs encoding wild-type human p53 and Hdm2 in pCMV and the p53 reporter plasmid pG13Luc were a gift from B. Vogelstein. The HA-tagged wild-type p53, p53 K305N (nuclear localization signal (NLS)) (17), and p53 173L (DNA binding-defective) mutants were provided to us by K. Maki and K. Vousden; Hdm2 NLS and Hdm2 G58A mutants were obtained from A. Levine. The pcDNA3.1 Myc-tagged Hdmx constructs (wild type and N-terminal truncation mutant

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¹ The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; HA, hemagglutinin; NLS, nuclear localization signal; GFP, green fluorescent protein.

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(amino acids 101–490), also designated Hdmxp53bs⁻) were described previously (15) and kindly provided by D. George. Full-length Hdmx and the N-terminal truncated (amino acids 101–490) Hdmx fragment were released from the above constructs using a *Bam*HI-*Xho*I restriction digest and subcloned into the pcDNA3X(+)/MyEGFP to generate Myc-GFP-tagged Hdmx and Myc-GFP-Hdmx-(101–490). The tagged Hdmx (R-NLS (amino acids 2–430) and R⁻ (amino acids 2–451)) deletion mutants, deleted either of the entire RING-finger region including the putative NLS or most of it to leave the putative NLS intact, were generated by PCR amplification of the corresponding fragments using a 5'-primer including a *Bam*HI site and 3'-primers including *Xho*I sites. The PCR products were subcloned into pcDNA3.1Myc and pcDNA3X(+)/MyEGFP to generate the Myc-tagged Hdmx (R-NLS) and the Myc-tagged Hdmx (R⁻), and the Myc-GFP-tagged Hdmx (R-NLS) and the Myc-GFP-tagged Hdmx (R⁻), respectively. Hdmx NLS⁻ mutants (KRPRD → ATPLD) were generated using the QuikChange mutagenesis kit (Stratagene). The following oligonucleotide and its complement were used for the mutagenesis in the context of the Myc-tagged Hdmx and Myc-GFP-tagged Hdmx constructs: 5'-tgtgagcacaaccactagacggg.

Tissue Culture and Immunofluorescence—U2OS, Saos-2, NIH-3T3, and Phoenix (derived from the 293T cell line) cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and 100 μg/ml penicillin and streptomycin. Transfections were carried out using the calcium phosphate method or the FuGENE transfection reagent (Roche Molecular Biochemicals). For immunofluorescence staining, cells were transfected at 80% confluency on glass coverslips in six-well dishes and fixed 24 h after addition of the DNA mixtures. Fixed coverslips were washed twice in phosphate-buffered saline, permeabilized in 0.15% Triton X-100 for 5 min, and incubated in blocking buffer (phosphate-buffered saline + 2% bovine serum albumin) for 30 min. Cells were then incubated in blocking buffer containing primary antibody for 1 h as indicated below and then washed extensively in phosphate-buffered saline before incubation with the appropriate fluorochrome-conjugated secondary antibody for 1 h. Stained cells were mounted on glass slides and examined using standard immunofluorescence or confocal microscopy. Alexa or Jackson ImmunoResearch secondary antibodies were used for confocal or standard microscopy, respectively.

Staining of p53 was carried out using the anti-p53 polyclonal FL-393 or anti-p53 monoclonal DO-1 (Santa Cruz Biotechnology), or, to detect HA-p53, the anti-HA polyclonal antibody HA.11 (Babco) was used. Mdm2 staining was carried out using the anti-Mdm2 monoclonal antibody SMP14 or the polyclonal antibody N20 (Santa Cruz Biotechnology). The Myc-tagged Hdmx proteins were detected using an anti-Myc (9E10) monoclonal or polyclonal (Upstate Biotechnology) antibody and/or the previously described anti-Hdmx mouse monoclonal antibody 6B1A (11) kindly provided by A. Jochemsen.

Luciferase Assay—U2OS cells were grown on six-well plates and transfected as outlined above. Luciferase analyses were performed with a commercial kit (Promega), and activity was measured in a luminometer. To correct for transfection efficiency, 1 μg of CMVlacZ was co-transfected in all cases, and luciferase values were corrected for β-galactosidase activity.

Immunoprecipitation and Western Blotting—Cells were rinsed with phosphate-buffered saline and scraped in the lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 2 mM dithiothreitol, 0.1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, 0.4 units/ml aprotinin, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM NaVO₄) + 10% glycerol and sonicated 2 × 7 s (Sonicator, Ultrasonic processor XL, Heat Systems, 12–14% power). Debris were removed by sedimentation at 4 °C (20 min at 14,000 rpm). For immunoprecipitation assays, cell lysates (~250 μg of protein) were incubated with 1–3 μg of the appropriate antibody (9E10 or SMP14) for 2 h at 4 °C. Following the addition of 30 μl of a protein A-Sepharose beads mixture, the reactions were incubated for 1 h at 4 °C; the beads were then washed three times in the lysis buffer and resuspended in 30 μl of SDS sample loading buffer. The immunoprecipitates or the protein extracts (for straight Western blot analysis) were resolved by SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell). The proteins of interest were detected by incubation of the membrane with primary specific antibody as indicated below in 5% milk followed by a horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody and a chemiluminescent substrate (ECL, Amersham Biosciences, Inc.). The membrane was probed with either an anti-HA monoclonal antibody (HA.11 from Babco) to detect the transfected p53 proteins (wild type and K305N) or the anti-p53 monoclonal antibody (DO-1). The Mdm2 antibody SMP14 (Santa Cruz Biotech-

nology) was used to detect Hdm2, and an anti-Myc (9E10) monoclonal antibody was used to detect the Myc-tagged Hdmx proteins.

RESULTS

Hdm2 Recruits Hdmx into the Nucleus—Determination of the subcellular localization of transfected Hdmx and/or Mdmx in different cellular contexts has led to conflicting observations (13, 18–21). To revisit this issue, we determined the localization of exogenous Myc-tagged (N-terminal) Hdmx and GFP-Hdmx in U2OS, Saos-2, and NIH-3T3 cell lines by immunofluorescence. In all cell types analyzed, both Hdmx-tagged proteins were mainly found in the cytoplasm (Fig. 1, *a–d*). The intensity and the frequency of the nuclear staining slightly varied from one cell line to another but always appeared much less frequent (<5% of cells) and less intense than the cytoplasmic staining (Fig. 1*h*).

Sequence analysis of Hdmx reveals a single putative NLS in the C-terminal RING-finger domain (KRPRD). Point mutations in this motif (KTLLD) had no major effect on the cellular distribution of Hdmx (Fig. 1, *e* and *h*). Deletion of the C-terminal RING domain instead resulted in both nuclear and cytoplasmic localization of Hdmx (Hdmx R-NLS and R⁻, carrying deletions of either the entire RING domain, including the putative NLS, or most of it to leave the putative NLS intact) (Fig. 1, *f–h*). It appears, therefore, that the putative NLS of Hdmx is not functional and that its C-terminal RING-finger motif is an important determinant of the cellular distribution of Hdmx.

Since Hdm2 is a nuclear cytoplasmic shuttling protein (2, 3) and Mdm2 (the mouse ortholog of Hdm2) forms stable heterooligomers with Mdmx (14, 15), we investigated whether Hdm2 levels affect Hdmx localization. Strikingly, GFP-Hdmx was entirely nuclear when co-expressed with Hdm2 in U2OS (Fig. 2, *c–e* and *o*) or Saos-2 cells (not shown). Similar results were obtained using a Hdmx-Myc tagged construct (not shown). This effect did not depend on the ability of Hdm2 to bind p53 since the p53 binding-defective mutant, Hdm2 G58A (22), was still able to recruit Hdmx into the nucleus (Fig. 2*o*).

More importantly this effect depends on the ability of Hdm2 to interact with Hdmx. Indeed, deletion of the RING domains of Hdm2 (Hdm2R⁻) or Hdmx (Hdmx R⁻ and R-NLS) abrogated their ability to form heterodimers (Fig. 2*p*) and the nuclear recruitment of Hdmx by co-expressed Hdm2 (Fig. 2, *f–k* and *o*).

Moreover, the putative NLS motif of Hdmx appears to be dispensable for its recruitment into the nucleus (Fig. 2*o*), whereas this effect is dependent on the integrity of the Hdm2 NLS (Fig. 2, *l–n* and *o*) (3) further supporting that the observed effect on Hdmx localization is mediated by a direct interaction with Hdm2.

Since p53 binds Hdmx and possesses a nuclear cytoplasmic shuttling activity independent of Hdm2 (23, 24), we tested whether p53 can also recruit Hdmx in the nucleus. As shown, overexpression of wild-type p53 had a minor, yet reproducible, delocalization effect on Hdmx (Fig. 2*o*). Expression of a p53 DNA binding-defective mutant (173L) was unable to alter the Hdmx localization pattern (Fig. 2*o*), thus suggesting that the slight nuclear accumulation of Hdmx following p53 overexpression is caused by the increased expression of a p53 target gene. Based on the data above, we propose that this effect is mediated by the transcriptional activation of endogenous Hdm2.

Hdmx Increases Hdm2 Protein Levels and Inhibits Hdm2-mediated p53 Ubiquitination—It is generally accepted that Hdm2-mediated ubiquitination of p53 is essential for its degradation. Since binding of both Mdmx and Hdmx to Mdm2 increases the steady-state level of Mdm2 without affecting p53 stability (13, 15), one obvious possibility is that Hdmx binding inhibits the Hdm2 E3 ligase function. This is supported by the

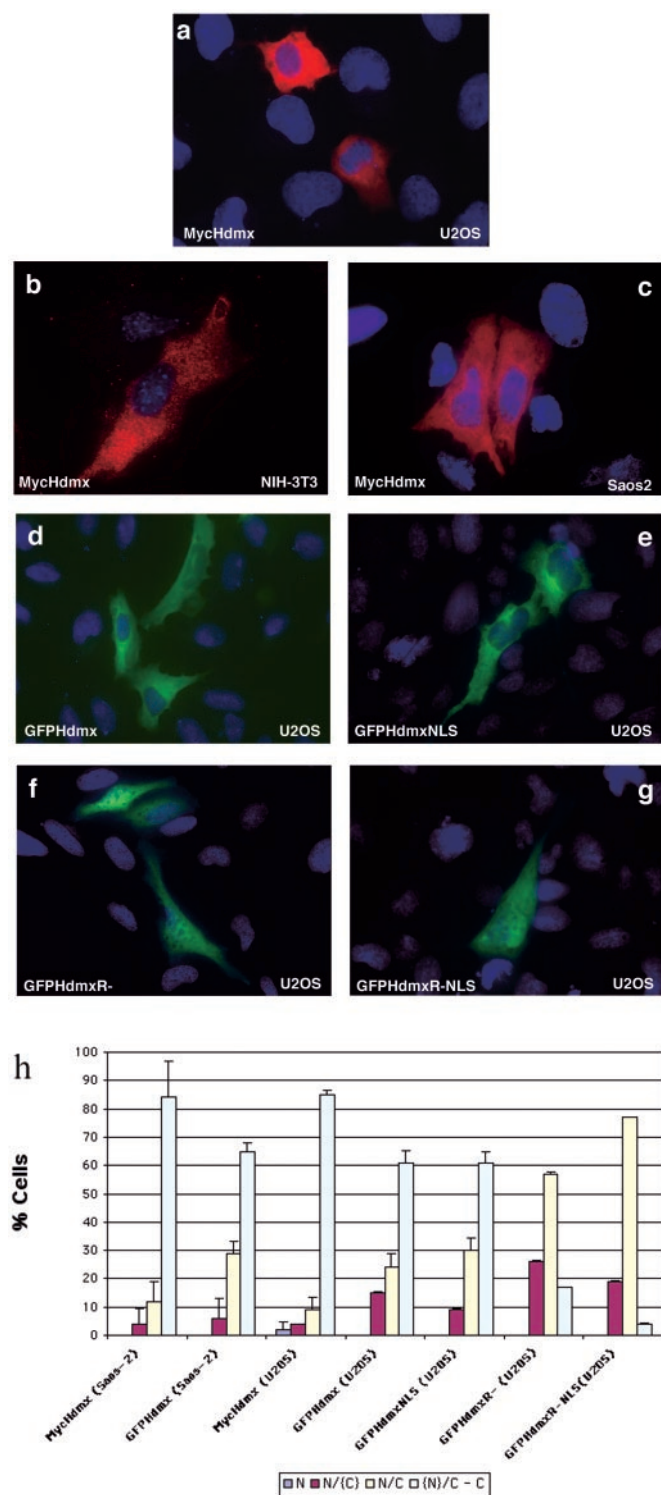


FIG. 1. Cellular localization of exogenous Hdmx wild type and mutants in NIH-3T3, Saos-2, and U2OS cells. *a-c*, U2OS, Saos-2, and NIH-3T3 cells were transfected with a plasmid expressing Myc-tagged Hdmx wild type alone. *d-g*, U2OS cells were transfected with a plasmid expressing GFP-Hdmx wild type (*d*), an NLS- mutant (*e*), and two C-terminal truncated mutants, R- (*f*) and R-NLS (*g*). After 24 h, cells were fixed and stained for Hdmx (red) using the anti-Myc monoclonal antibody 9E10 (*a-c*) by indirect immunofluorescence. Cells were examined for Cy3 fluorescence (*a-c*) and for GFP-Hdmx fluorescence (green) (*d-g*). DNA (blue) is stained with 4,6-diamidino-2-phenylindole (*a-g*). *h*, the Hdmx staining pattern in cells (Saos-2 and U2OS) transfected with the indicated Hdmx constructs was scored for more than 100 cells in two separate experiments; values are means \pm S.D. Cells were scored as having fluorescence exclusively in the nucleus (N) or cytoplasm (N/C), or stronger in the nucleus (N/(C)), equal in the nucleus and cytoplasm (N/C), or stronger in the cytoplasm (N/C - C).

finding that Hdmx contacts Hdm2 in its RING-finger domain, a region essential for its ubiquitin ligase activity (6, 7).

We therefore investigated the levels of p53 ubiquitination in the presence of overexpressed Hdm2 and Hdmx by immunoblotting analysis (Fig. 3). As expected, overexpressed Hdm2 promoted ubiquitination of endogenous p53. This effect was dose-dependent (Fig. 3, lanes 2 and 3) and based on the ability of Hdm2 to bind p53 (G58A mutant, Fig. 3, lane 4). Expression of Hdmx instead had no measurable effect on p53 ubiquitination (Fig. 3, lane 5), indicating that Hdmx by itself does not have ubiquitin ligase activity toward p53. Co-transfection of Hdmx and Hdm2 resulted in stabilization of the Hdm2 protein, as expected, that was dependent on the integrity of the Hdmx RING domain (Fig. 3, compare lanes 2 with 6, 7 and 2 with 8, 9). In these conditions, Hdmx did not detectably change the overall pattern of Hdm2-dependent ubiquitination of p53. However, in evaluating the effect of Hdmx on Hdm2-dependent p53 ubiquitination, one should consider the property of Hdmx to increase the protein levels of Hdm2. To this end, we set up experimental conditions that allowed the same amount of Hdm2 protein to be expressed even in the presence of increasing Hdmx protein concentrations (Fig. 3, lanes 10 and 11). Under these conditions, a significant reduction of p53 ubiquitination was observed in the presence of high Hdmx protein levels. Notably, expression of the HdmxR- mutant did not significantly interfere with Hdm2-mediated p53 ubiquitination (Fig. 3, lanes 8 and 9). Similar results were obtained analyzing the status of ubiquitination of exogenous p53 in different cellular contexts such as in U2OS, Saos-2, and p53xMdm2 knockout murine embryonic fibroblasts (HA-p53) (not shown). Together these data indicate that Hdmx has the potential to inhibit Hdm2 ligase activity. However, the concomitant ability of Hdmx to stabilize the Hdm2 protein suggests that, under physiological conditions, inhibition of the Hdm2 ligase activity by Hdmx is not sufficient to account for the inhibition of Hdm2-mediated p53 degradation by Hdmx.

Hdmx Blocks Hdm2-mediated Nuclear Export of p53—It is well documented that wild-type p53 must reach the cytoplasm to be efficiently degraded (2, 4, 5, 24, 25). An alternative explanation for the stabilization of p53 is, therefore, that Hdmx interferes with the Hdm2-dependent p53 nuclear export. To test this possibility, we investigated the reciprocal effects of Hdm2, Hdmx, and p53 on their subcellular localization.

Overexpressed p53 was almost exclusively nuclear and, when expressed with Hdm2, was partly relocated to the cytoplasm as expected (4, 5) (Fig. 4, *a-f* and *s*). Strikingly, co-expression of Hdmx inhibited the Hdm2-induced cytoplasmic delocalization of p53 (Fig. 4, *g-j* and *s*), indicating that nuclear Hdmx can block the Hdm2-dependent nuclear export of p53. p53 export, however, was not efficiently perturbed by Hdmx when co-expressed with the Hdm2 NLS mutant. Importantly we have shown earlier that the nuclear recruitment of Hdmx by Hdm2 is only partial. Mutants of Hdmx unable to bind Hdm2, such as Hdmx R- and R-NLS, were incapable of reversing the Hdm2-induced export of p53 (Fig. 4, *k-n* and *s*) even if a significant fraction of these mutants are found in the nucleus (see also Fig. 1, *f-h*). Together these observations indicate that the nuclear localization of Hdmx is necessary but not sufficient to reverse Hdm2-induced nuclear export and that this effect is dependent on the ability of Hdmx to interact with Hdm2.

Finally it has been shown that nuclear export of p53 is not required for its ubiquitination and that a p53 cytoplasmic mutant (K305N) can be ubiquitinated and degraded by Hdm2 NLS (26). Our data suggest that stabilization of p53 by Hdmx is the consequence of its ability to block p53 nuclear export. Therefore, Hdmx should be unable to reverse the Hdm2-dependent

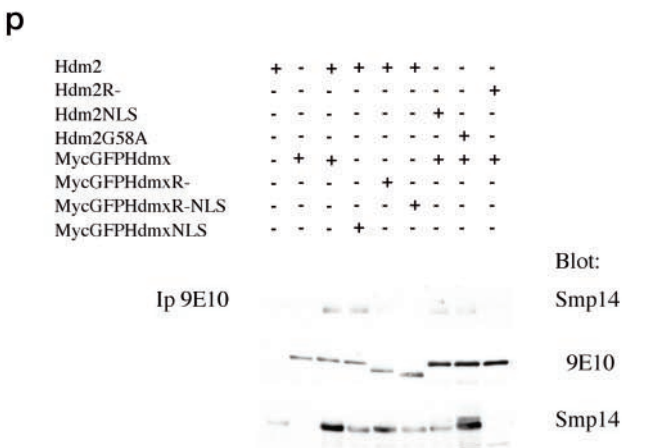
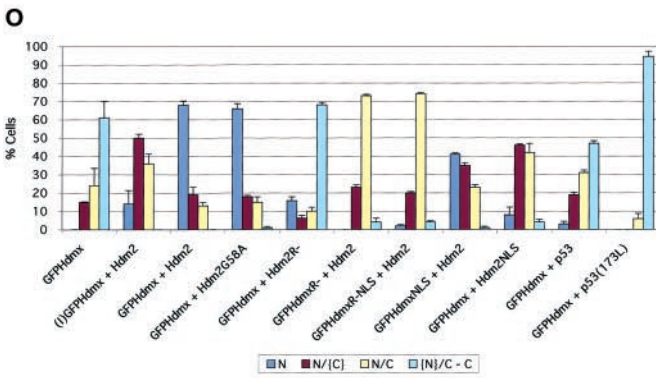
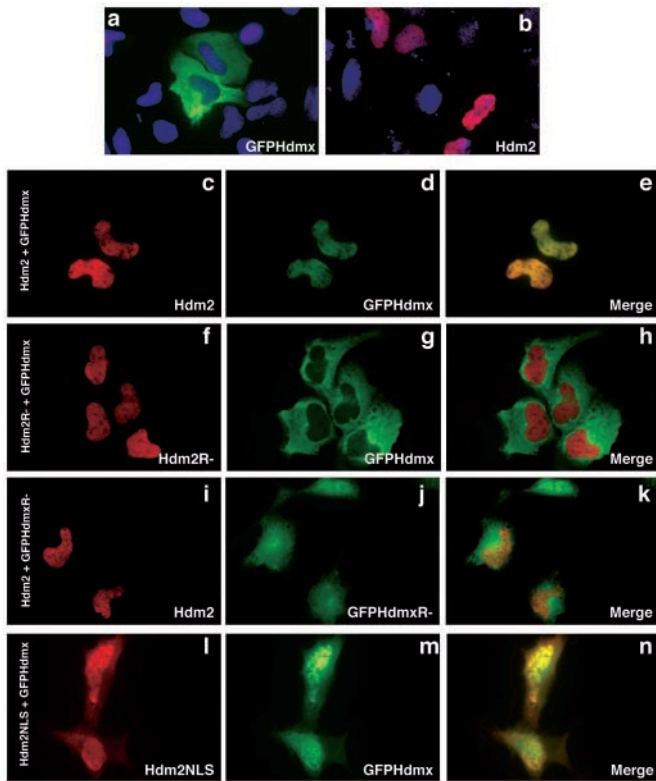


FIG. 2. Cellular redistribution of exogenous Hdmx wild type and mutants upon Hdm2 or p53 co-expression. *a-f*, U2OS cells were transfected with a plasmid expressing GFPHdmx (*a*) and Hdm2 (*b*) wild type alone or with plasmids expressing GFPHdmx and Hdm2 (*c-e*), GFPHdmx and Hdm2R- (*f-h*), GFPHdmxR- and Hdm2 (*i-k*), or GFPHdmx and Hdm2 NLS (*l-n*). After 24 h, cells were fixed and stained

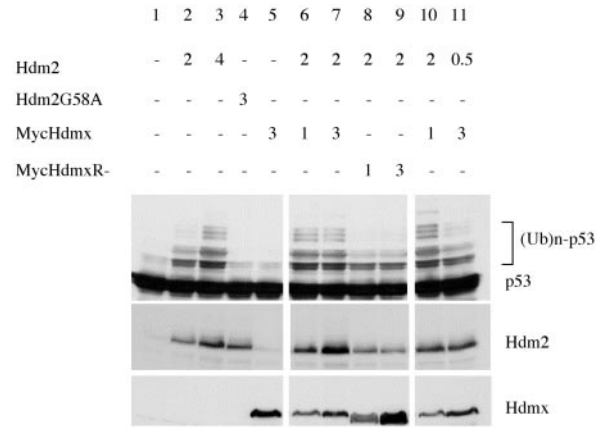


FIG. 3. Effects of Hdmx on Mdm2-mediated p53 ubiquitination. Phoenix cells were transfected with DNAs encoding Hdm2 wild type and mutants alone (*lanes 2, 3, and 4*) or in the presence of either Myc-GFPHdmx wild type (*lanes 6, 7, 10, and 11*) or Myc-GFPHdmxR- (*lanes 8 and 9*). Cells were also transfected with DNA expressing Myc-GFPHdmx alone (*lane 5*). The amount of DNA (μg) transfected is indicated. *Lanes 1* correspond to nontransfected cells. The protein extracts were prepared 24 h later and examined by immunoblot analysis with the p53 monoclonal antibody DO-1. The ladder of bands indicated is endogenous ubiquitinated p53 ((*Ub*)n-p53). Hdm2 and Hdmx protein levels were monitored in the transfected cells by stripping the blot and reprobing with the Mdm2 monoclonal antibody SMP14 and the anti-Myc monoclonal antibody 9E10.

degradation of cytoplasmic p53. To test this hypothesis, we examined the levels of p53 and p53(K305N) following overexpression of Hdm2 or Hdm2 NLS, either alone or in the presence of Hdmx, in the U2OS (Fig. 5, *a* and *b*) or Soas-2 cells and in the p53xMdm2 knockout murine embryonic fibroblasts. In the absence of Hdmx, Hdm2 and Hdm2 NLS induced ubiquitination (not shown) and subsequent degradation of p53 or p53(K305N), respectively. However, co-expression of Hdmx appeared to stabilize wild-type p53 but had no effect on the p53 cytoplasmic mutant, indicating that the ability of Hdmx to inhibit Hdm2 function is restricted to the nuclear pool of Hdmx. In contrast, Hdmx expression resulted in the stabilization of both Hdm2 and Hdm2 NLS-. This last observation suggests that the Hdmx-dependent stabilization of Hdm2 and p53 are achieved through different mechanisms.

Nuclear Hdmx Down-regulates p53-dependent Transcription—Overexpression of Hdmx results in a modest, yet consistent, decrease of the p53 transcriptional regulation ability (11–13, 20). At first analysis, this effect of Hdmx on p53-dependent transcription contrasts with our observation that overex-

pression of Hdm2 (*red*) using the anti-Mdm2 monoclonal antibody SMP14 by indirect immunofluorescence. Cells were examined for Cy3 fluorescence and for GFP-Hdmx fluorescence (*green*). DNA (*blue*) is stained with 4,6-diamidino-2-phenylindole. *o*, the GFPHdmx pattern in U2OS cells transfected with the indicated GFPHdmx, Hdm2, and p53 constructs was scored for more than 100 cells in three separate experiments; values are means \pm S.D. Cells were scored as having fluorescence exclusively in the nucleus (*N*), stronger in the nucleus (*N/(C)*), equal in the nucleus and cytoplasm (*N/C*), or stronger in the cytoplasm (*(N)/C-C*). Only cells expressing both Hdmx and Hdm2 proteins were considered for the analysis. (For *I*, 0.5 μg of GFPHdmx was transfected with 0.25 μg of Hdm2, and for all the other transfection conditions, 0.5 μg of GFPHdmx-expressing constructs was transfected with 1 μg of Hdm2- or p53-expressing constructs.) *p*, co-immunoprecipitation of Hdmx species with wild-type or mutant Hdm2 proteins. Lysates from U2OS cells transfected with the indicated constructs were treated with anti-Myc antibody (9E10) and protein A beads. Immunoprecipitates were analyzed by Western blotting using anti-Hdm2 antibody (*upper panel*). Comparable Hdmx and Hdm2 transfection efficiencies were confirmed by Western blotting of lysate samples using anti-Myc antibody (*middle panel*) and anti-Hdm2 antibody (*lower panel*). The Hdm2 protein levels are increased in the presence of wild-type Hdmx. *I*p, immunoprecipitation.

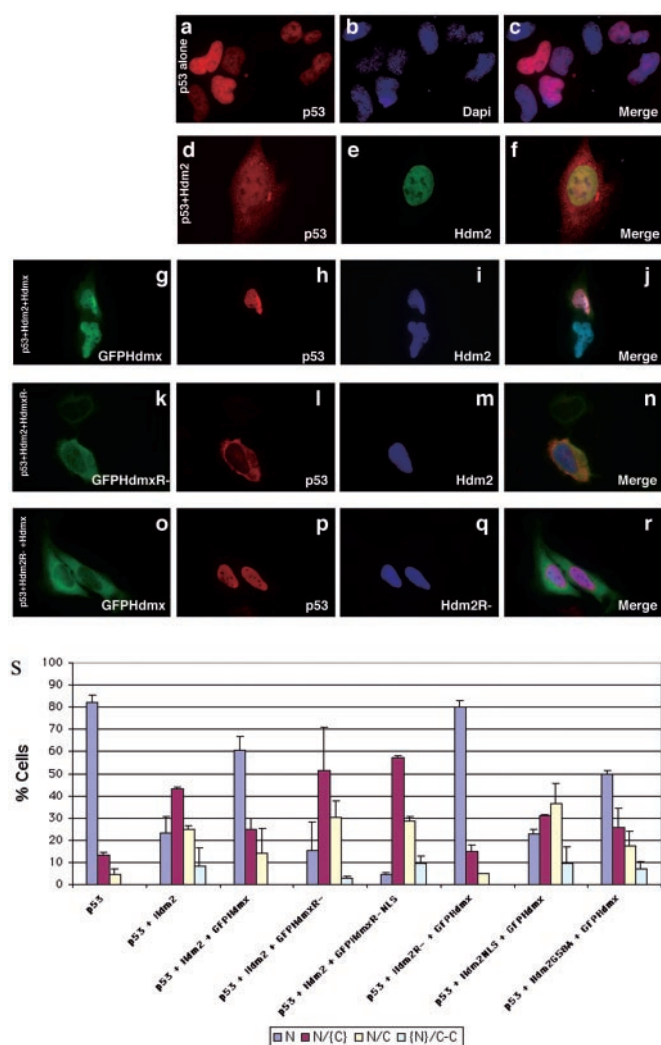


FIG. 4. Hdm2 does not relocalize p53 in the presence of Hdmx. *a-r*, U2OS cells were transfected with a plasmid expressing p53 alone (*a-c*) or with plasmids expressing p53 and Hdm2 (*d-f*); p53, Hdm2, and GFPHdmx (*g-j*); p53, Hdm2, and GFPHdmxR- (*k-n*); or p53, Hdm2R-, and GFPHdmx (*o-r*). After 24 h, cells were fixed and stained for p53 (red) Hdm2 (blue) using the anti-p53 polyclonal antibody FL393 and the anti-Mdm2 monoclonal antibody SMP14 by indirect immunofluorescence. Cells were examined for Cy3 and Alexa (blue) fluorescence and for GFP-Hdmx fluorescence (green). *s*, the staining pattern for p53 in U2OS cells expressing the indicated GFPHdmx, Hdm2, and p53 constructs were scored for more than 100 cells in three separate experiments; values are means \pm S.D. Cells were scored as described in Fig. 2o. Only cells expressing the three proteins were considered for the analysis. Hdmx does not prevent Hdm2-NLS-induced p53(K305N) degradation. Dapi, 4,6-diamidino-2-phenylindole.

pressed Hdmx is mainly localized to the cytoplasm. However, in all reported experiments but one (12), the effect of Hdmx was investigated by transient transfection using various p53-deficient cell lines (Hep3B, H1299, Calu, and p53^{-/-} murine embryonic fibroblasts), overexpressed p53, and various reporter constructs (14, 15). In these experimental conditions, overexpressed p53 might trigger recruitment of Hdmx into the nucleus indirectly through its effect on the endogenous *Hdm2* promoter. To test this possibility, we have transfected U2OS cells, which express endogenous p53, with the reporter construct pG13Luc containing 13 copies of the p53 consensus sequence upstream of a minimal promoter driving transcription of the luciferase gene. As expected, endogenous p53 is able to sustain a significant basal level of expression of the reporter construct (Fig. 6). Overexpression of Hdm2, but not of Hdmx, significantly inhibited pG13Luc transcription. Expression of

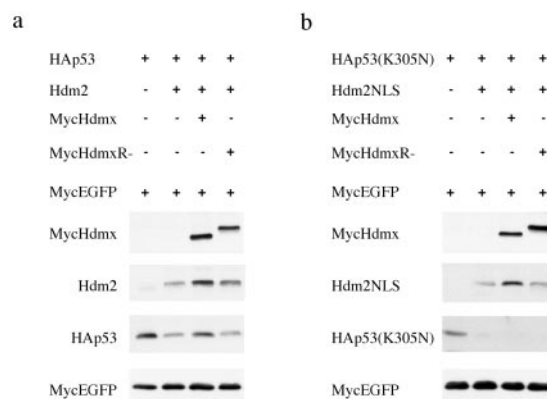


FIG. 5. Hdmx does not reverse Hdm2-NLS-mediated degradation of cytoplasmic p53(K305N). *a*, U2OS cells were transfected with 2 μ g of HA-p53 alone or together with 5 μ g of Hdm2 in the absence or presence of either 5 μ g of Myc-GFPHdmx or 5 μ g of Myc-GFPHdmxR-. *b*, U2OS cells were transfected with 2 μ g of HA-p53(K305N) alone or together with 5 μ g of Hdm2-NLS in the absence or presence of either 5 μ g of Myc-GFPHdmx or 5 μ g of Myc-GFPHdmxR-, all in combination with 1 μ g of Myc-EGFP. Cell lysates were analyzed by immunoblot analysis with the anti-HA monoclonal antibody (HA.11) to detect p53 levels, with the anti-Hdm2 SMP14 antibody to detect the Hdm2 proteins, and with the anti-Myc antibody (9E10) to detect the Hdmx proteins. Equal transfection efficiencies were confirmed using the anti-Myc antibody (9E10) for the detection of Myc-EGFP.

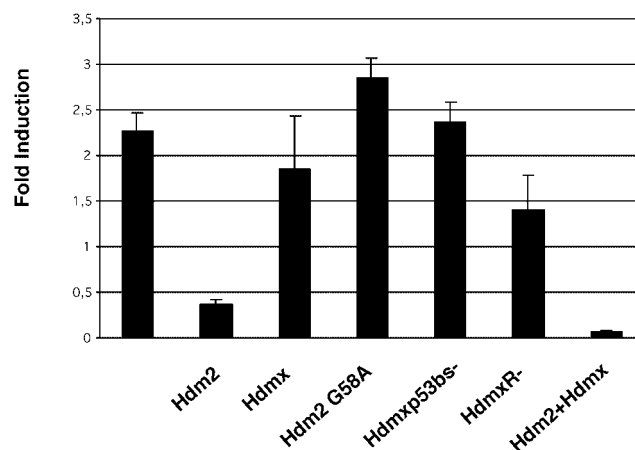


FIG. 6. Hdm2-dependent nuclear recruitment of Hdmx is necessary for efficient inhibition of p53 transcriptional activity. U2OS cells were transfected with 400 ng of the p53 reporter construct (pG13Luc) and DNAs expressing Hdm2 wild type and p53 binding-defective mutant G58A (300 ng), GFP-Hdmx wild type, p53 binding-defective mutant (Hdmxp53bs-), and RING domain-deleted mutant (HdmxR-) alone (800 ng) or a combination of both wild-type proteins as outlined in the transfection scheme. Luciferase activity is shown as fold induction compared with no transfection of the reporter with the S.D. of triplicates.

HdmxR-, which is partly localized in the nucleus (see Fig. 1, *f* and *h*), slightly, yet reproducibly, reduced the p53 transcriptional activity. Strikingly, simultaneous expression of Hdm2 and Hdmx resulted in an almost complete repression of p53 pG13Luc transcription. These findings demonstrate that regulation of Hdmx by Hdm2 is an absolute requirement for the efficient regulation of p53 activity by Hdmx and suggest that only the nuclear fraction of Hdmx regulates p53-dependent transcription.

DISCUSSION

We have shown here that exogenous Hdmx, when expressed alone, is mainly cytoplasmic, while, in the presence of Hdm2, it becomes mainly nuclear. Nuclear relocalization of Hdmx by overexpressed Hdm2 depends on their reciprocal interaction as

suggested by the finding that this phenomenon requires the integrity of the RING domain of both proteins. Surprisingly, however, RING mutants of Hdmx (R- and R-NLS) were found in both the nucleus and cytoplasm even when expressed alone suggesting that Hdmx binds other cellular factor(s) that might sequester Hdmx in the cytoplasm in the absence of Hdm2. Since Hdm2 binds Hdmx in the same RING-finger region, it might compete for binding and displace this putative interaction. Alternatively, formation of Hdmx oligomers might occur in the cytoplasm and inhibit the nuclear import of Hdmx by, for instance, masking its putative NLS, which is located in the RING-finger motif of the protein. In this context, Hdm2, which forms stable heterodimers with Hdmx (14), might disrupt the formation of Hdmx oligomers.

We then investigated the mechanisms by which nuclear Hdmx inhibits Hdm2-induced p53 degradation. Since this effect was previously reported to be mediated by the Hdmx RING-finger and to involve hetero-oligomerization with the Hdm2 RING-finger, we hypothesized that Hdmx inhibits the ubiquitin ligase activity of Hdm2. Our data, however, are not entirely consistent with this model. Indeed, as it was demonstrated earlier for p19^{ARF} (27), we showed that the interaction with Hdmx reduces the ubiquitin ligase activity of Hdm2. However, this effect is counteracted *in vivo* by the ability of Hdmx to stabilize Hdm2 suggesting that Hdmx stabilizes p53 through other mechanisms. And in fact, Hdmx is able to rescue Hdm2-mediated degradation even in those experimental conditions where the overall levels of p53 ubiquitination are not affected by Hdmx. The block of the Hdm2 ligase activity by Hdmx might instead explain the stabilization of Hdm2 following Hdmx overexpression. In fact, Hdm2 is not only a ubiquitin protein ligase for p53 but also mediates its own ubiquitination and stabilization (6).

It is believed that the nuclear export of p53 is critical for efficient Hdm2-mediated degradation, although the function of Hdm2 in p53 nuclear export is still a matter of debate. We have demonstrated that Hdmx antagonizes Hdm2-dependent p53 relocalization, thereby providing a simple model for the ability of Hdmx to stabilize p53. However, the mechanisms through which Hdmx inhibits nuclear export of p53 by Hdm2 remain elusive. It has been shown that mutation of the Hdm2 RING-finger impairs the ubiquitination and cytoplasmic relocalization of p53, suggesting that ubiquitin ligation is a crucial signal for p53 export (4, 5). Considering the modest effect of Hdmx on Hdm2-induced p53 ubiquitination, Hdmx must exert its stabilization function more downstream by counteracting the relocalization effect of the ubiquitination signal. Hdmx forms a ternary complex with Hdm2 and p53 (11) and could, for instance, occlude the C-terminal p53 nuclear export signal. This signal has, indeed, been proposed to be indispensable for Hdm2-mediated p53 export (4, 5). Alternatively, overexpressed Hdmx might compete with Hdm2 for p53 binding. This possibility, however, appears unlikely since Hdmx does not significantly disturb binding of Hdm2 to p53 in co-immunoprecipitation experiments (11).²

Finally we confirmed that Hdmx antagonizes p53 transcriptional activity and demonstrated that nuclear recruitment of Hdmx is essential for its ability to suppress efficiently p53 transactivation. Indeed, in our experimental setting, the effect of Hdmx on p53-dependent transcription was marginal when expressed alone, while it became dramatic when co-expressed with Hdm2. The mechanism for this suppression is unknown. The formation of a trimeric complex could block crucial interactions with proteins of the transcriptional machinery.

The biological significance of these particular functions of

Hdmx, however, remains to be elucidated. Recent reports, including observations presented here, indicate that stabilized p53 protein is not able to activate transcription, and it was proposed that Hdmx secures the presence of a pool of inactive p53 that can be instantly activated when needed. Overexpressed Hdmx, however, is an artificial situation, and it is not known whether or not it corresponds to any physiological situations. Indeed, Hdmx protein levels seem to be very low in all primary cells tested. On the other hand, increased Hdmx levels were found in a significant fraction of tumor cell lines when compared with normal cells (28). In general Hdmx expression in these tumor cell lines correlates with the presence of wild-type p53, suggesting that deregulated expression of Hdmx plays a role in carcinogenesis as an alternative way to inactivate p53. High levels of Hdmx may also explain the efficient response to radiation and chemotherapy displayed by a subset of tumors, including germ-cell tumors, which express high levels of wild-type p53 and Hdm2 (29, 30). The now available mouse models further support the notion that, at least during embryogenesis, the primary function of Hdmx is to inhibit p53 function (16).²

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