Hdmx Modulates the Outcome of P53 Activation in Human Tumor Cells*[s]

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Tumors that express wild-type P53 provide a target for therapies designed to reactivate P53 function. This is supported by the potent activation of *P53* in tumor cells by Nutlin, a cis-imidazoline that inhibits the Hdm2-P53 interaction. The efficacy of Hdm2·P53 antagonists could be compromised if they do not antagonize Hdmx, an Hdm2 homolog that inhibits P53 transactivation. We evaluated the role of Hdmx expression in sensitivity to Nutlin in a range of cancer cell lines. Nutlin reduced Hdmx levels in normal cells and some cancer cell lines, whereas other cancer cells were refractory to such down-regulation. Strikingly, Nutlin did not disrupt Hdmx·P53 complexes, and in cell lines where no Hdmx degradation occurred, Nutlin failed to induce apoptosis. shRNA-mediated reduction of Hdmx sensitized cells to apoptosis, but caspase-3 was neither required nor sufficient for Hdmx degradation or apoptosis. Our data imply that Hdmx is an important determinant of the outcome of P53 activation. Thus, targeting Hdmx may be a therapeutic strategy that complements drugs such as Nutlin.

Novel tumor regression strategies include those designed to target cancer-specific molecular alterations. Drugs that inhibit pathways involved in proliferation, inhibition of cell death or metastasis are attractive candidates for targeted therapy. Conversely, small molecules that trigger apoptosis in tumor cells also hold great promise as novel chemotherapeutics. The P53 tumor suppressor is a transcription factor that can induce cell death or growth arrest via induction of numerous target genes (1, 2). The cell cycle arrest and pro-apoptotic functions of P53 are critical for tumor suppression, in part explaining why *p53* is mutated in up to 50% of human cancers. However, the frequency at which *p53* is mutated varies between tumors of different origins and within tumor types according to clinical stage (3–5). Therefore, in a substantial fraction of tumors there is no mutation of *p53* itself, and re-activation of P53 function in these cancers represents a viable strategy for inducing tumor regression.

Genetic studies indicate that the ubiquitin ligase H/Mdm2 is a major negative regulator of P53 (where H and M denote the human and murine homologs, respectively). *Mdm2* knock-out is lethal, and is rescued by concomitant deletion of *p53* (6, 7). The two main functions of Hdm2 that attenuate P53 both involve Hdm2 binding to P53. First, Hdm2 can antagonize P53-mediated transactivation by binding the P53 N-terminal transactivation domain (8). Second, Hdm2 is an E3 ubiquitin ligase that targets P53 for proteasome-mediated degradation (9, 10), though the mechanisms remain to be elucidated (11). Because the *Hdm2* gene is P53-responsive, an autoregulatory loop is set up in which P53 levels are kept low in non-stressed conditions by Hdm2-mediated degradation.

The Hdm2-related protein, Hdmx, is also a critical regulator of P53 activation. Like *Mdm2* knock-out, *Mdmx* deficiency is also lethal in a P53-dependent manner (12). Although Hdmx can bind to both Hdm2 and P53 (13, 14), Hdmx is not a ubiquitin ligase, and is not a P53-regulated gene (14, 15). Therefore, some of the molecular mechanisms by which Hdmx suppresses P53 activity are likely to be distinct from those utilized by Hdm2. Current data indicate that Hdmx can inhibit P53 transcriptional activity *in vitro*, and recent data from mouse models suggest that Mdmx is a more significant inhibitor of P53 transactivation than Mdm2 *in vivo* (16, 17). This may occur directly, because of Hdmx binding to the P53 transactivation domain, or indirectly via stimulation of Hdm2-dependent inhibition of P53 (18, 19). Because both Hdm2 and Hdmx are overexpressed in many tumors that retain wild-type *p53*, they are appealing targets for therapeutic approaches.

Numerous stresses perturb the P53/Hdm2 autoregulatory loop and lead to P53 stabilization, either by physical separation of Hdm2 from P53 or by inhibiting Hdm2-mediated P53 ubiquitination. For example, genotoxic insult activates damage-responsive kinases, such as ataxia telangectasia-mutated (ATM)[5] protein (reviewed in Ref. 20). ATM activates P53 in part by direct phosphorylation of P53 itself and also by destabilization and inhibition of Hdm2 (21). ATM-dependent phosphorylation also triggers degradation of Hdmx in an Hdm2-dependent manner (22, 23). Therefore, DNA damage potently stimulates P53 activity by eliminating interactions with its two main negative regulators. However, the use of genotoxins in chemother-

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[5] The abbreviations used are: ATM, ataxia telangectasia-mutated; NCS, neocarzinostatin; Q-PCR, quantitative PCR; FACS, fluorescent-activated cell sorting.

apy is associated with undesirable side effects including nausea, alopecia and induction of secondary malignancies (24, 25). This is presumably because of induction of damage, as well as activation or repression of a plethora of other signaling pathways in normal and tumor cells by clastogenic agents.

Recently, Vassilev *et al.* (26) developed a cis-imidazoline compound, Nutlin-3a, which binds to Hdm2 and inhibits its interaction with P53, resulting in increased P53 levels and transcriptional activity. Importantly, Nutlin-induced P53 activation is not associated with induction of DNA damage or damage-induced modifications of P53 (27). As such, Nutlin represents a lead compound in the search for drugs that can specifically activate the P53 pathway in cancer.

Recent findings indicate that Nutlin may be particularly effective in cancers that overexpress Hdm2 (28). However, Nutlin is also able to induce apoptosis in myeloma and leukemia cells that do not overexpress Hdm2 (29–31). The effectors of Nutlin-induced, P53-dependent cell cycle arrest or apoptosis may vary according to cell and tumor type. For example, cell cycle arrest may be induced by p21CIP, a p53-responsive gene commonly up-regulated following Nutlin treatment (26, 28). shRNA-based screening has also implicated 53BP1, a P53-binding protein, in Nutlin-induced cell cycle arrest (32). Additionally, there are indications that Nutlin increases levels of p53-responsive pro-apoptotic genes *PUMA*, *PIG3*, and *bax* in B cell chronic lymphocytic leukemia cells (30, 33). Still, the functional significance of each of these genes in Nutlin-induced apoptosis is unclear. Similarly, very little is known about factors that may be involved in preventing some cancer cells from undergoing Nutlin-induced apoptosis, with the exception of *p53* mutation.

Here, we show that Hdmx level can determine the biological response to Nutlin treatment. We find that high Hdmx level, or an inability to degrade Hdmx, can contribute to Nutlin resistance. Conversely, reduction of Hdmx can sensitize cells to Nutlin-induced, P53-dependent apoptosis. While Hdmx reduction did significantly increase activation of some P53 target genes, apoptotic genes such as *Bax* and *PUMA* were not among them. Additionally, while Hdm2 and Hdmx are structurally related in their P53 binding regions, we also show that Nutlin does not prevent Hdmx from binding to P53. These data indicate that Hdmx level is an important factor for the optimization of genotype-specific tumor therapies, and they suggest that Hdmx may affect the output of the P53 pathway by mechanisms both related to and independent of P53 transactivation.

EXPERIMENTAL PROCEDURES

Cell Culture and Drug Treatments—MCF7 and 293T cells were cultured in Dulbecco's modified Eagle's medium/10% fetal bovine serum with Ciprofloxacin. WS1 (normal human fibroblasts) and BJ-TERT (telomerase-immortalized normal human fibroblasts) were cultured in MEM/15% fetal bovine serum supplemented with non-essential amino acids (Invitrogen, 1:50 dilution), vitamins (Invitrogen, 1:50 dilution), and β-mercaptoethanol (Sigma, 10 μM final concentration). MCF7 cells stably expressing caspase-3 were a kind gift from Dr. Reiner Jänicke (University of Dusseldorf, Dusseldorf, Germany). BL2, BL40, and BL41 (Burkitt lymphoma cells, a kind gift from Dr. Martin Allday, Imperial College, London,

UK) were cultured in RPMI/10% heat-inactivated fetal bovine serum with penicillin/streptomycin. Neocarzinostatin (stock 1 mg/ml in sodium acetate) was obtained from Robert Schultz at NCI, the proteasome inhibitor MG132 was from Calbiochem and Nutlin-3a was a kind gift from Lyubomir Vassilev (Hoffman-La Roche, Nutley, NJ). Cycloheximide (US Biologicals) was dissolved in 0.1% ethanol and used at a final concentration of 100 μg/ml.

Western Blotting, Immunoprecipitation, and Antibodies— Cells were lysed in radioimmune precipitation assay buffer with CompleteMini protease inhibitors (Roche Diagnostics), phenylmethylsulfonyl fluoride, sodium vanadate, and sodium fluoride. Following SDS-PAGE, proteins were transferred to polyvinylidene difluoride membrane (Millipore, MA). The following antibodies were used for Western blotting: P53, DO1 (Calbiochem, mouse, 1:1000) or FL393 (Santa Cruz Biotechnology, rabbit, 1: 1500); Phospho-Ser[15] P53, phosphospecific anti-phospho-Ser[15] (Cell Signaling Technology, rabbit, 1:1000); Hdmx, mouse monoclonal 8C6 (1:100 overnight), a kind gift from Jiangdong Chen, or BL1258 (Bethyl Laboratories, 1:10000, overnight); Hdm2, triple mouse monoclonal mixture of IF-2 (Calbiochem), SMP-14 (Santa Cruz Biotechnology) and 4B2(Calbiochem) (1:500 each, overnight); p21, C-19 (Santa Cruz Biotechnology, 1:1500); actin, (rabbit, 1:20000, Sigma); full-length caspase-3, (rabbit, 1:1000, Cell Signaling Technology). Peroxidase-conjugated secondary antibody was used at 1:10,000 (Jackson Immunochemicals). For P21 immunofluorescence, monoclonal antibody from Transduction Laboratories, KY, was used at 1:400.

For immunoprecipitation analyses, cells were harvested on the plate in lysis buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 150 mM NaCl. 0.5% Nonidet P-40, with phenylmethylsulfonyl fluoride, sodium fluoride, and Complete protease inhibitor added immediately prior to use). Typically, 500 μg to 2 mg of protein were used for immunoprecipitation. The antibodies used were: DO1 or agarose bead-conjugated FL393 (P53), p55 rabbit polyclonal (for Hdmx, a kind gift of Aart Jochemsen), and IF2 (Hdm2). 2 μg of each antibody per milligram of protein was used, with the exception of p55 where 3 μl of the serum was used. Immobilized recombinant protein A was used for antibody pull-down, and immunoprecipitates were washed and eluted as described (21).

SYBR Green Quantitative PCR (Q-PCR)—Total RNA was prepared using QiaShredder and RNeasy RNA isolation kits per the manufacturer's instructions (Qiagen). 2 μg of total RNA per sample was used for cDNA synthesis with random hexamer primers, using SuperScriptIII Reverse Transcriptase system (Invitrogen). Real-time quantitative PCR was performed using an ABI PRISM 7700 Sequence Detection System, using Platinum SYBR SuperMix (Invitrogen) with ROX as an internal standard. Changes in gene expression were normalized to *18 S* mRNA. Primer sequences are included in the Supplementary Data.

Lentiviral-mediated Hdmx Knockdown—Double-stranded DNA oligonucleotides (Invitrogen) encoding the Hdmx knockdown construct, or a negative control shRNA targeting chicken MKP3 (a kind gift of Yasuhiko Kawakami) were ligated into pSUPER vector (a gift of R. Bernards) and subcloned into
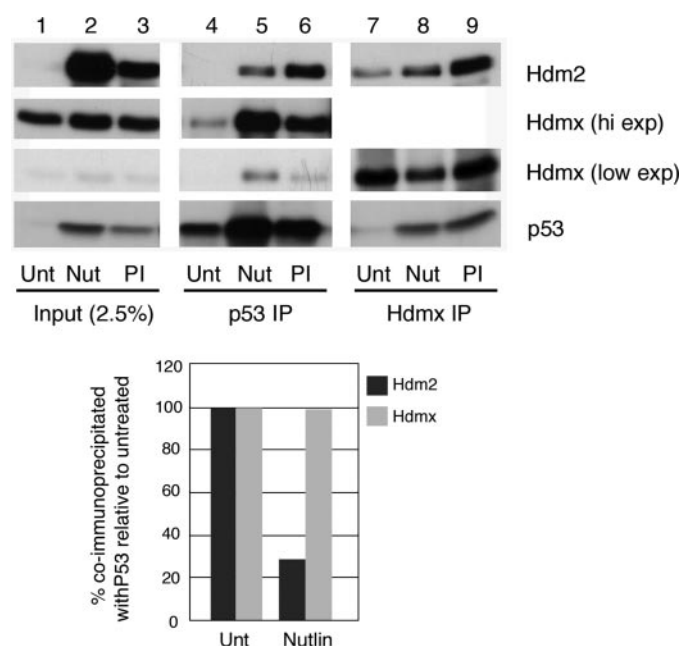
FIGURE 1. **Nutlin separates Hdm2, but not Hdmx, from P53.** MCF7 were treated with 10 μM Nutlin or 10 μM MG132 for 5 h. 2 mg of total lysate was used as input for both P53 and Hdmx immunoprecipitations. *Lanes 4–6* are the P53 immunoprecipitates. *Lanes 7–9* are the Hdmx immunoprecipitates. High film exposure for Hdmx *lanes 7–9* is omitted because it is overexposed. The graph indicates the relative amount of Hdm2 and Hdmx that co-immunoprecipitated with P53, with untreated amounts set to 100%.

FIGURE 2. **Nutlin induced Hdmx down-regulation in fibroblasts is associated with decreased Hdmx half-life and increased P53 activity.** *A*, kinetics of P53, Hdm2 and P21 induction and Hdmx down-regulation in BJ fibroblasts following treatment with NCS (50 ng/ml) or 5 μM Nutlin. *B*, cells were treated with 100 ng/ml NCS (5 h), or with 5 μM Nutlin (24 h) prior to addition of cycloheximide (*CHX*) to determine Hdmx half-life.

pCPPT-GFP vector (34). shRNA sequences are included in the Supplementary Data. For knockdown in MCF7, we infected parental MCF7 cells with lentivirus expressing shRNA against Hdmx or against MKP3. Lentivirus was produced by Lipofectamine2000 transfection of 293T cells at 90% confluence on 10-cm plates with the following: 10 μg of lentivirus-Hdmx, 5 μg of pMDLg/pRRE, 2.5 μg of RSV-Rev, and 3 μg of MD2.VSVG. Virus-containing supernatant was harvested 48–72 h post-transfection and pooled before filtering through a 0.45-μm filter. Aliquots were made and flash-frozen prior to titration in the target cell line by fluorescence-activated cell sorting. Cells were infected by plating 10^5 cells in a 6-well plate and performing three rounds of infection in a total volume of 2 ml with 7 μg/ml polybrene.

Apoptosis Measurements—Floating and adherent cells were harvested and apoptosis was quantified by propidium iodide staining and flow cytometry to detect the sub-G$_1$ population. Images of cells before and after treatments were taken using a Nikon Coolpix camera mounted on a Nikon Eclipse TS100 light microscope.

RESULTS

Nutlin Does Not Disrupt Hdmx·P53 Complexes—Nutlin is a potent P53 activator in the cell lines analyzed thus far and was identified based on its ability to inhibit Hdm2-P53 interactions. Because Hdmx and Hdm2 have similar, though not identical, amino acids that interact with P53 (19, 35), it is possible that Nutlin also prevents Hdmx-P53 interaction. We tested this by co-immunoprecipitation experiments in MCF7 cells as they express significant amounts of Hdmx (Fig. 1). In unstressed cells, a small fraction of Hdmx was bound to P53 (lane 4). In
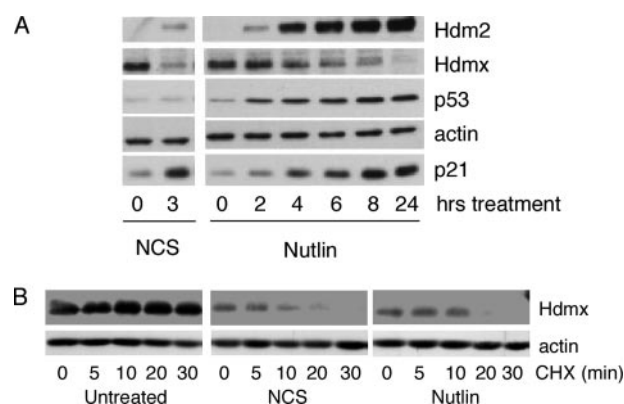
order to evaluate the binding of Hdm2 to P53, we used the proteasome inhibitor MG132 to stabilize P53/Hdm2 complexes (lane 6). We next compared the ability of Nutlin to affect the association of Hdmx or Hdm2 with P53. Nutlin treatment stabilized P53 and increased Hdm2 to levels exceeding those achieved by proteasome inhibition (compare lanes 2 and 3, input). Nutlin treatment reduced the amount of Hdm2 co-immunoprecipitated with P53 to 20–30% the level observed in the proteasome-inhibitor treated controls (compare lanes 2 and 5 with lanes 3 and 6). Strikingly, the opposite was observed for the Hdmx-P53 interaction. After Nutlin treatment, more Hdmx was found in complex with P53 compared with cells treated with proteasome inhibitor (compare lanes 2 and 5 with lanes 3 and 6). Reciprocal co-IP using Hdmx antibody confirmed these results (Fig. 1, *lanes 7–9*). Together these data indicate that Nutlin preferentially disrupts Hdm2·P53 complexes and that disruption of Hdmx·P53 complexes is not required for robust activation of P53 by Nutlin. These *in vivo* observations are supported by *in vitro* Biacore binding studies showing that the K_d of Nutlin-3a for Hdm2 is 47 nM, whereas for Hdmx it is greater than 10 μM (data not shown).

Hdmx Is Down-regulated by Nutlin in Normal Human Fibroblasts—These data raise the question of whether Nutlin activates P53 maximally, because it cannot antagonize the binding of Hdmx, an important negative regulator of P53 transactivation (16, 17, 36). Recent data show that Hdmx is subject to Hdm2-mediated degradation following DNA damage (37). It is possible that the increases in Hdm2 following Nutlin treatment could similarly lead to Hdmx degradation. In this way, Nutlin induction of Hdm2 would create a positive feedback loop for P53 activation via degradation of Hdmx. We tested this possibility in two normal fibroblast strains, BJ and WS1. As observed previously, P53 activation following DNA damage induced by the radiomimetic agent neocarzinostatin (NCS) was associated with an increase in Hdm2 levels and a decrease in Hdmx (Fig. 2A and Ref. 23). Strikingly, we observed similar effects when BJ or WS1 normal human fibroblasts were treated with Nutlin (Figs. 2A and 4A); as Hdm2 levels rose with increasing times of Nutlin treatment, Hdmx steady state levels decreased as a consequence of its shorter half-life (Fig. 2B). *Hdmx* mRNA did not
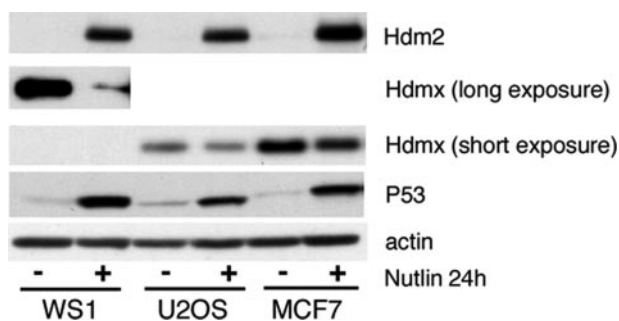
FIGURE 3. **Hdmx down-regulation is not required for Nutlin-induced p53 activation.** WS1, MCF7, and U2OS were treated with 10 μM Nutlin for 24 h prior to Western blotting. Low and high exposures are shown for Hdmx to illustrate the difference in expression levels, and the down-regulation of Hdmx in WS1 cells.

change following Nutlin treatment (data not shown). Consistent with previous reports, Nutlin did not induce DNA damage (supplemental Fig. S1). These data imply that in normal fibroblasts increasing Hdm2 level alone is sufficient to lead to Hdmx degradation and P53 activation.

Nutlin-induced Hdmx Degradation Can Be Attenuated in Transformed Cells—We next determined whether the same type of positive feedback loop might occur in tumor cells in which Hdmx levels are higher than normal, or in which the Hdm2:Hdmx ratio is aberrant. Such a mechanism could explain how Nutlin activates P53 in cells with high Hdmx levels, despite its inability to disrupt Hdmx·P53 complexes. We used U2OS and MCF7 cells for these studies as they express more Hdmx than normal cells. Fig. 3 shows that in U2OS and MCF7 cells, Nutlin significantly induced Hdm2, but Hdmx degradation was attenuated. Note also that Nutlin substantially increases P53 level in these cells, consistent with its ability to stabilize P53 by preventing Hdm2-P53 binding. These studies show that the Hdm2 mass-driven degradation of Hdmx observed in normal cells can be attenuated during transformation, but that Nutlin is still able to activate P53 (also see Fig. 4*C*).

The Impact of Hdmx Levels on P53 Activation in Normal and Cancer Cells—We infer that P53 can be activated in cells in which Hdmx is not degraded because Nutlin increases P53 to levels exceeding the Hdmx level available for inhibition. To evaluate the extent to which Hdmx can mitigate Nutlin-induced P53 transactivation, we analyzed induction of the P53 target gene *p21*. We first evaluated normal human fibroblasts in which Hdmx is degraded in response to Nutlin treatment. Analyses were done at saturating drug doses and times by using 5 and 10 μM Nutlin for either 5 or 24 h. In addition to Western analysis, p53 target gene induction was measured by real time Q-PCR. As Fig. 4*A* shows, within 5 h of treatment at two Nutlin doses, there was no measurable decrease in Hdmx level, while P53 and Hdm2 were both increased, and P53 transactivation was significantly elevated. By 24 h, Hdmx was undetectable, and P53 transactivation increased a further 2–3-fold. Consistent with recent analyses (16, 17), these data imply that Hdmx can modulate P53 transcriptional output. However, they also show that reducing Hdmx level is not required for Nutlin-mediated activation of P53.

We next analyzed the effects of Nutlin treatment on P53 transactivation in U2OS and MCF7 cells. This experiment ana-
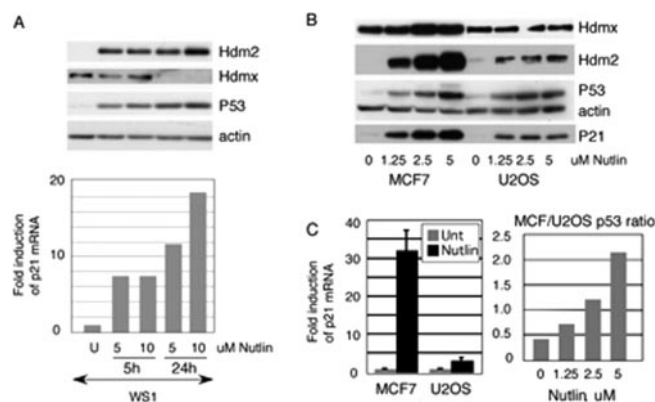

FIGURE 4. **Comparison of Nutlin-induced P53 activation in WS1 (normal human fibroblasts), MCF7 and U2OS.** *A*, WS1 were treated with 5 or 10 μM Nutlin for 5 and 24 h, and analyzed by Western blot. P21 mRNA was analyzed by Q-PCR. *B* and *C*, MCF7 and U2OS cells were treated for 5 h with the indicated dose of Nutlin, and analyzed for expression of Hdmx, Hdm2, P53, and P21 by Western blot or Q-PCR. Note the significantly higher induction of P21 and Hdm2 in MCF7.

lyzed the effects of increasing Nutlin dosage for a fixed time (5 h) corresponding to the onset of P53 transactivation. Fig. 4*B* shows that whereas the P53 levels resulting from Nutlin treatment are similar, the levels of P53 target genes are profoundly different, with MCF7 cells exhibiting far higher levels of *Hdm2* and *p21* than U2OS cells. The Q-PCR analysis in Fig. 4*C* demonstrates that P53 transactivation is at least 10-fold higher in MCF7 than in U2OS, even though the amount of P53 in MCF7 relative to U2OS cells is only 2-fold higher after Nutlin treatment (Fig. 4*C*). Note also that Hdmx is not degraded at this time point in either cell type (Fig. 4*B*). These data demonstrate that while Hdmx level is one determinant of P53 activation following Nutlin treatment, there are other factors that profoundly affect P53 transactivation in transformed cells.

Hdmx Can Modulate the Biological Response to Nutlin— Nutlin invariably induces P53-dependent cell cycle arrest, whereas the amount of apoptosis depends on the cell type (28). While proteins such as P21 and 53BP1 can contribute to arrest (28, 32), factors that modulate Nutlin-induced apoptosis remain unclear. Mdmx deficiency *in vivo* can lead to apoptosis (38, 39), and we observed attenuated down-regulation of Hdmx in some cancer cell lines (Fig. 3). Therefore, we evaluated whether Hdmx levels may also determine the biological response to Nutlin. We first examined Burkitt lymphoma cell lines expressing wild type (BL2 and BL40) or mutant P53 (BL41). Fig. 5 indicates that Nutlin treatment of BL40 and BL2 cells lead to increased P53 levels at 5h (lanes 2, 3, 8, 9). The transcriptional activity of P53 also increased in both cell lines, as measured by *p21* mRNA induction (Fig. 5*A*, *lower panel*). Notably, *p21* induction was higher in BL2 compared with BL40 at the 5-h time point, despite lower levels of P53 protein. We observed that Hdmx was rapidly downregulated in BL2 cells, but unaffected in BL40 cells throughout the time course. The defect in Hdmx degradation in BL40 appears to be specific to Nutlin treatment, because DNA damage induced marked Hdmx down-regulation in these cells (Fig. 5*A*, *lane 6*). These data extend our observations of attenuated Hdmx down-regulation to a tumor type of different (hematologic) origin.
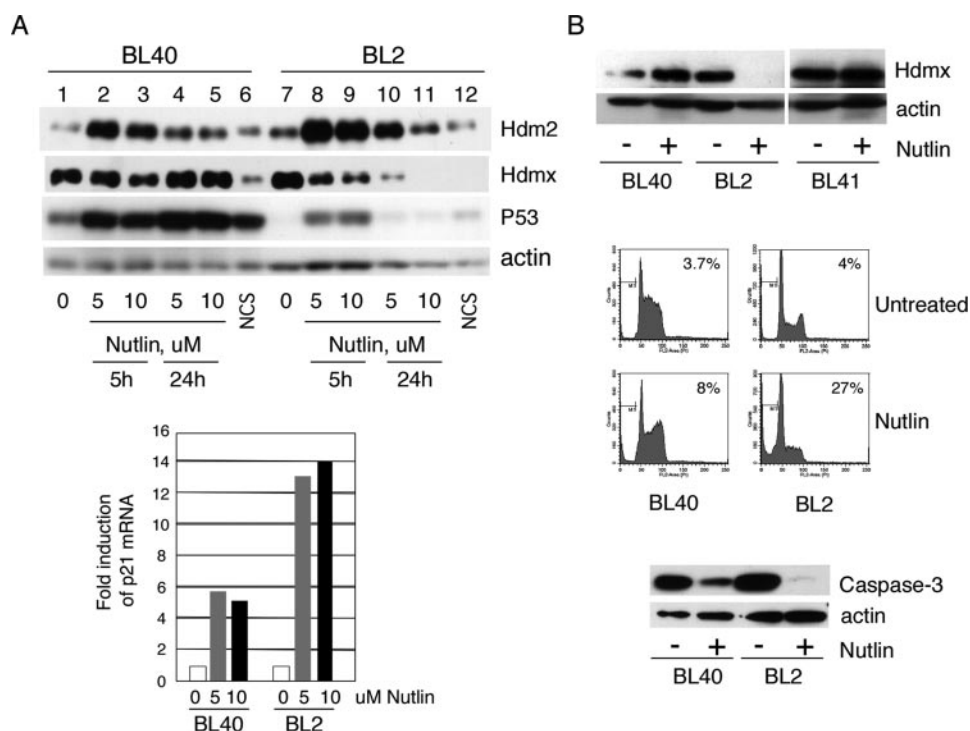
FIGURE 5. **Hdmx down-regulation correlates with induction of apoptosis in BL.** *A*, BL40 or BL2 were treated with 5 or 10 μM Nutlin for the indicated times, or with 100 ng/ml NCS for 5 h prior to analysis by Western blot. Note the robust down-regulation of Hdmx in BL2 compared with BL40. *Lower panel* shows p21 mRNA induction after 5 h of Nutlin treatment at the indicated doses. *B*, *upper panel*: BL40 and BL2 (wild type P53) and BL41 (mutant P53) were treated with Nutlin for 48 h and analyzed for Hdmx levels by Western blot or subjected to FACS analysis following propidium iodide staining (*middle panels*). Percentage of subG$_1$ (apoptotic) cells is indicated for each profile. *Lower panel*, BL40 and BL2 were analyzed for cleavage of full-length caspase-3 after 24 h of Nutlin treatment.

whether reduction of Hdmx, increased P53 transcriptional activity, or both, contribute more to the phenotypic outcome.

To investigate this, we examined the biological response of MCF7 cells to Nutlin. These cells were chosen since P53 transactivation is high following Nutlin treatment (Fig. 4), yet Hdmx is not down-regulated. Fig. 6 indicates that parental MCF7 cells arrest in response to Nutlin treatment, in agreement with recent reports (28, 32). These data are consistent with the notion that a failure to down-regulate Hdmx is associated with resistance to Nutlin-induced apoptosis. MCF7 cells lack caspase-3 expression because of a deletion in exon 3 (41). Our results in BL cells indicated that *caspase-3* was not sufficient for Nutlin-induced apoptosis (Fig. 5). However, MCF7 and BL cells are from different lineages, and we considered it possible that caspase-3 could be required for apoptosis in a lineage-specific manner. To test this, we treated MCF7 cells stably expressing caspase-3 (MCF7-c3) with Nutlin. Fig. 6 shows that the response of MCF7-c3 was identical to that of parental MCF7 cells, as they also underwent cell cycle arrest rather than apoptosis. Furthermore, we did not observe caspase-3 activation following Nutlin treatment in MCF7-c3 (Fig. 6 and supplemental Fig. S4). Together with the results in BL cells, our data suggest that neither caspase-3 expression nor activation is sufficient to trigger Hdmx degradation or apoptosis in response to Nutlin. Additionally, these data indicate that other factors determine the sensitivity of MCF7 cells to Nutlin-induced death.

Hdmx Knockdown Sensitizes to Nutlin-induced Apoptosis— Hdmx is a major negative regulator of P53, and this can occur via inhibition of P53 transactivation function (16, 17, 36). Since Hdmx degradation was attenuated in MCF7 cells after Nutlin treatment, we evaluated whether knockdown of Hdmx could sensitize them to Nutlin-induced death. Fig. 7 indicates that lentiviral delivery of *Hdmx* shRNA lead to ~60% knockdown of *Hdmx* mRNA and protein (Fig. 7*A* and data not shown). Virtually 100% of cells were infected using this method (supplementary data Fig. S3). This amount of knockdown did not affect P53 basal levels (Fig. 7*A*).

The immunoprecipitation data in MCF7 cells (Fig. 1) suggest that Nutlin does not effectively disrupt Hdmx·P53 complexes. If these complexes limit Nutlin-induced P53 transactivation, a prediction is that Hdmx knockdown should enhance P53 activity. To test this, we treated MCF7 cells infected with control or *Hdmx* shRNA with Nutlin. Fig. 7, *A*

We also evaluated the apoptotic response of BL40 and BL2 to Nutlin. Fig. 5*B* shows that Hdmx was not down-regulated in BL40, and these cells were less sensitive to Nutlin-induced cell death than were BL2, in which Hdmx was rapidly down-regulated. The number of sub-G$_1$ cells in BL2 at this time point (48 h) is an underestimate of apoptosis, as virtually all cells showed signs of apoptosis by light microscopic analysis by 24 h (supplementary data Fig. S2). The rapid induction of apoptosis may also explain the decrease in Hdm2 and P53 in BL2 at later time points (Fig. 5*B*, *lanes 10* and *11*). BL41 cells, which express a transactivation-deficient P53 (R248Q), were resistant to apoptosis and also failed to down-regulate Hdmx following Nutlin treatment (Fig. 5*B* and supplementary data, Fig. S2). This demonstrates that P53 transactivation is required for Nutlin-induced Hdmx down-regulation, most likely via increased Hdm2 levels as described above.

We observed that caspase-3 was activated in BL40 and BL2 cells, yet the latter were significantly more sensitive to apoptosis (Fig. 5*B*). This result is interesting, because Hdmx is reportedly cleaved following caspase activity (40). Our data indicate that caspase-3 activation is not sufficient for Hdmx degradation in BL40 cells. Furthermore, they suggest that caspase-3 activation is insufficient for maximal induction of apoptosis in response to Nutlin. Together our results in BL cells indicate that Nutlin-induced cell death correlates with down-regulation of Hdmx and increased P53 transactivation, rather than caspase-3 activation. However, these data do not indicate
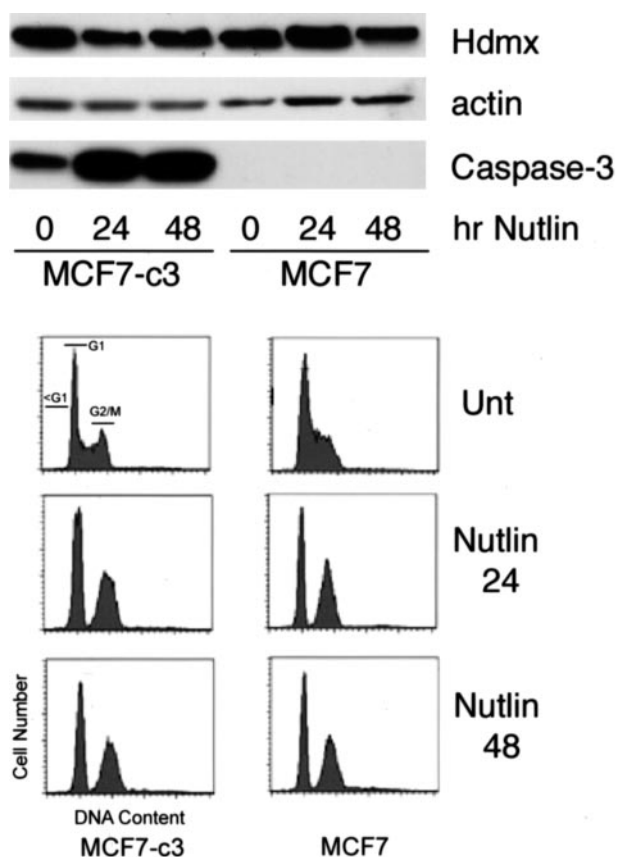
FIGURE 6. **Reconstitution of caspase-3 does not sensitize MCF7 to Nutlin.** *Upper panel,* MCF7 or MCF7 expressing caspase-3 were treated with 10 μM Nutlin for 48 h and analyzed by Western blot. *Lower panel,* FACS analysis of propidium iodide-stained cells.

and *B* indicate that Hdmx knockdown slightly enhanced P21 induction following Nutlin treatment at the protein and RNA level. These data are consistent with Hdmx being a negative regulator of p53 transactivation function. However, when we extended our analysis to other p53 target genes, a more complex pattern emerged. Fig. 7*B* shows that Hdmx knockdown did not enhance the transactivation of *bax* or *PUMA*, or the recently identified p53 target, *DRAM* (42). We also analyzed induction of *TIGAR*, a p53 target implicated in modulation of apoptosis (43). *TIGAR* was not up-regulated in MCF10A cells expressing dominant negative p53 (*DD*, Fig. 7*B*), confirming that it is a P53 target gene. Similarly to *p21*, there was an enhancement of *TIGAR* induction following Hdmx knockdown, although the increase was small (Fig. 7*B*). Together, these data indicate that Hdmx knockdown appears to have a differential effect on stress-induced activation of p53 gene targets.

In parallel with the quantitative PCR analyses, we examined the effect of Hdmx knockdown on sensitivity to Nutlin. We treated MCF7 cells with Nutlin in the presence of control or Hdmx shRNA. Fig. 7*C* indicates that Hdmx knockdown alone had a minor effect on MCF7 apoptosis (compare upper panels). The small increase was presumably due to the partial Hdmx knockdown (Fig. 7*A*) we consistently achieved with our method of shRNA delivery. Strikingly, Hdmx knockdown lead to a significant increase in Nutlin-induced apoptosis compared with

control shRNA (Fig. 7*C*, compare *bottom panels*). This was reflected in a lower number of cells available for analysis, and in trypan blue viability counts (data not shown). In summary, our results suggest that Hdmx can modulate both p53 transcriptional output and the biological response to Nutlin.

DISCUSSION

Targeted disruption of the Hdm2-P53 interaction for reactivation of wild-type P53 is a promising therapeutic strategy for the treatment of cancer (44). Here, we show that Nutlin has selectivity for disruption of Hdm2·P53 complexes and does not inhibit the Hdmx-P53 interaction. We also observe Hdmx degradation in response to Nutlin treatment, but find this destabilization is attenuated in some cancer cell lines. Although the effectors of P53-dependent apoptosis in response to Nutlin are presently unclear, our data suggest caspase-3 is dispensable for the process. Strikingly, we find that Hdmx down-regulation correlates with induction of apoptosis in some cancer cells, and that reduction of Hdmx levels can sensitize cells to Nutlin-induced cell death. Our data validate Hdmx as a target for chemotherapeutics, and emphasize the need to evaluate the relationship between Hdmx destabilization, subcellular localization and the biological response to stresses that activate P53.

Nutlin Does Not Disrupt Hdmx·P53 Complexes—Nutlin binds to Hdm2 and prevents its interaction with the N terminus of P53, leading to P53 stabilization and activation (26). Because Hdmx also binds P53 at the N terminus, it was logical to assume that Nutlin would also abrogate Hdmx-P53 binding. Strikingly, we find that Nutlin inhibits Hdm2, but not Hdmx from binding to P53 (Fig. 1). Our data confirm a recent study showing that Nutlin did not affect the Hdmx-P53 interaction (45). Thus, although Hdm2 and Hdmx share ~30% identity at the amino acid level, there appears to be sufficient changes in tertiary structure that allow Hdmx to bind P53. Similarly, other groups reported peptides that bind with higher affinity to Hdm2 than Hdmx and selectively inhibit the Hdm2-P53 interaction (35). Together, these data suggest that drugs designed to specifically disrupt the Hdmx-P53 interaction may prove useful in tumors overexpressing Hdmx. Such drugs might be considered as single agents, or in combination therapy with Hdm2·P53 antagonists such as Nutlin.

Nutlin Induces Hdmx Down-regulation in Normal Human Fibroblasts—Genotoxins induce modifications of P53, Hdm2, and Hdmx that lead to P53 activation (22, 23, 46, 47). Concomitant with P53 activation in many cell types, Hdmx is degraded by an Hdm2-dependent process (37). Here, we show that Nutlin treatment of normal fibroblasts also leads to Hdmx degradation. This correlates with P53 transcriptional activation, suggesting that removal of Hdmx contributes to Nutlin-induced P53 activity. In contrast to DNA damage, Nutlin treatment does not induce phosphorylation of P53 (supplementary Fig. S1 and Ref. 27). We infer that in normal cells, Nutlin-induced Hdm2 must exceed a certain threshold, above which Hdmx degradation occurs. In agreement with this, we have not observed Hdmx down-regulation after Nutlin treatment in cells expressing mutant P53, where Hdm2 is not induced (Fig. 5*B* and data not shown). These data support the proposal that p53-depend-
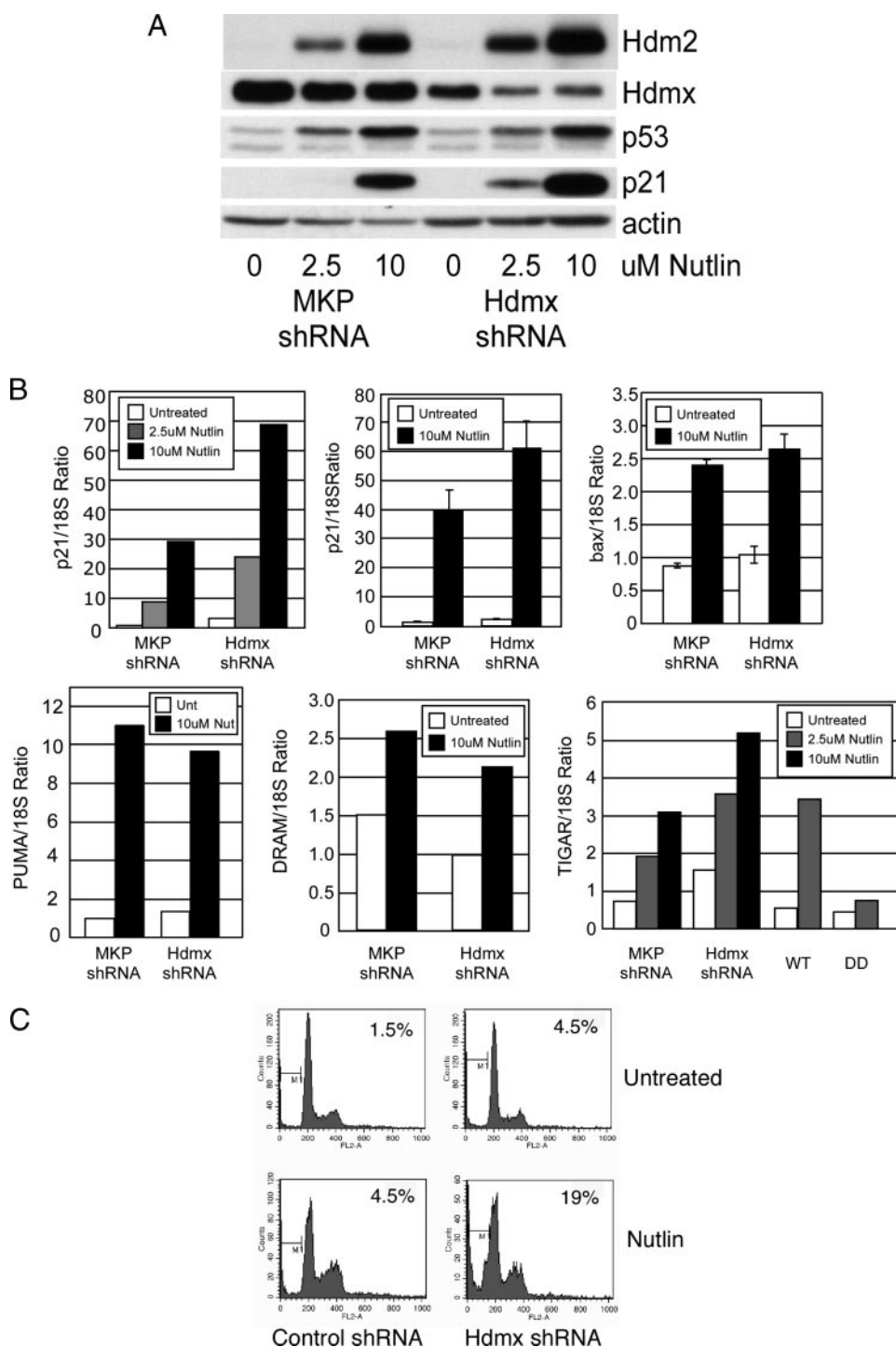
A

B

C

FIGURE 7. **Hdmx knockdown in cancer cells enhances Nutlin-induced cytotoxicity.** *A*, MCF7 were infected with lentivirus expressing control (MKP) or Hdmx shRNA, then treated with the indicated dose of Nutlin for 24h prior to Western blot analysis. *B*, MCF7 infected with lentivirus expressing shRNA against MKP or Hdmx were treated with 2.5 μM (*gray bars*) or 10 μM (*black bars*) Nutlin, or left untreated for 24 h prior to analysis of mRNA expression by Q-PCR. The first p21 graph is from the same experiment as the Western data in *A*, and the second summarizes the results of three independent experiments. *C*, MCF7 were infected with lentivirus expressing control of Hdmx shRNA for 48 h prior to treatment with 10 μM Nutlin and FACS analysis by PI staining.

MCF7 cells following Nutlin treatment. Because Hdmx levels are high in these cells, the amount of Hdm2 induced by Nutlin may be insufficient to trigger Hdmx degradation. Alternatively, other factors regulating Hdmx stability in these cells may be aberrant. We have also observed attenuated down-regulation of Hdmx in immortalized cells of epithelial and lymphoid origin, indicating the effect is not restricted to a particular cell type.[6] Because Hdmx was downregulated in BL2 cells, we cannot make a clear distinction between normal and tumor cells regarding Nutlin-induced Hdmx degradation. However, our observations in normal human fibroblasts and MCF7 cells have been confirmed in an independent study by Hu *et al.*[7] Future studies will determine the frequency at which Nutlin treatment fails to down-regulate Hdmx in cancer cells, and should uncover the responsible mechanisms.

There was a correlation between P53 transcriptional activation and Hdmx down-regulation in normal fibroblasts and BL2 lymphoma cells. One interpretation of these results is that Hdmx must be down-regulated in order to maximally activate P53. However, extension of our analyses to other cell lines indicates a more complex picture. For example, Nutlin-induced P53 transactivation is higher in MCF7 than in U2OS cells, despite slightly higher levels of Hdmx in the former cell line. These data indicate that Hdmx down-regulation is not invariably required for P53 transactivation following Nutlin treatment. Furthermore, they suggest cell-type specific factors determine the threshold for Nutlin-induced P53 activation. Our co-immunoprecipitation experiments show that in MCF7 cells, Hdmx is still bound to P53 at a time when P53 is transcriptionally active. If Hdmx is able to antagonize P53 transactivation, how can these observations be reconciled? Possibly, both Hdmx *and* Hdm2 binding to P53 are required for

ent induction of Hdm2 leads to Hdmx degradation (37, 45, 48), thus creating a positive feedback loop for p53 activation (45, 49, 50).

Hdmx Down-regulation Is Attenuated in Some Cancer Cells— We observed attenuated Hdmx down-regulation in U2OS and

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maximal transcriptional inhibition. This suggests a model in which Hdmx/Hdm2 binding constitutes the optimal transcriptional antagonist of P53 (18). However, this model is not consistent with recent data obtained *in vivo* (16, 17). Alternatively, after Nutlin treatment, the number of free molecules of P53 may exceed the amount of Hdmx in the nucleus, thereby allowing P53 to robustly transactivate its target genes. Ongoing quantitative analyses of the stoichiometry of P53 and its regulators in response to various stresses will be extremely informative in this regard.

Modulation of Nutlin-induced Apoptosis by Hdmx—Nutlin causes P53-dependent cell cycle arrest in most cell types examined to date, but Nutlin-induced apoptosis is cell type-dependent (28). If cell cycle arrest is not permanent, tumor regrowth could occur. Induction of apoptosis, on the other hand, ensures that tumor cells are eliminated. Therefore, targeting factors that block apoptosis in tumor cells would be of therapeutic benefit. To date, factors that modulate sensitivity to Nutlin-induced apoptosis remain unclear. We observed that MCF7 cells undergo cell cycle arrest following Nutlin treatment, in agreement with previous reports (28, 32). MCF7 cells also arrest following genotoxic stress, and this phenotype can be converted to apoptosis when caspase-3 is restored in these cells (52). However, we found that restoration of caspase-3 did not sensitize MCF7 to Nutlin-induced apoptosis. Furthermore, caspase-3 was not activated in these cells by Nutlin treatment. These data indicate that, unlike DNA damage (52), Nutlin does not trigger upstream activators of caspase-3 in MCF7 cells. We did not observe a strict correlation between activation of caspase-3 and Nutlin-induced apoptosis in Burkitt lymphoma. Therefore, we infer that although caspase-3 can be activated in response to Nutlin in some cell types, it is neither required nor sufficient to induce apoptosis.

By contrast, our data indicate that the lack of Hdmx degradation in MCF7 and BL40 cells does correlate with resistance to Nutlin-induced apoptosis. In agreement with this, knockdown of Hdmx in MCF7 cells sensitized them to Nutlin. We also observed protection against Nutlin-induced apoptosis when Hdmx was overexpressed in SJSA osteosarcoma cells that are otherwise extremely sensitive to Nutlin.[8] These data concur with a recent report showing that Hdmx overexpression blocks Nutlin-induced apoptosis in transformed human fibroblasts (45). It is important to note that Hdm2 can target other proteins for degradation, including Rb, TIP60, P21, and TCAP (53–56). Therefore Hdm2-dependent degradation of factors in addition to Hdmx may also modulate Nutlin-induced apoptosis.

The Effect of Hdmx Level on p53 Transactivation and the Response to Stress—Mdmx and Hdmx are critical negative regulators of p53 transactivation *in vivo* (16, 17, 57). Consistent with this, we observed that Nutlin induction of p21 levels was increased following Hdmx knockdown. Although this may contribute to the reduction in MCF7 cell number following the combination of Nutlin and Hdmx knockdown, it is unlikely to explain the increase in apoptosis. We did not observe changes in the expression of the proapoptotic genes *bax* and *PUMA*

following Hdmx knockdown; however, it is possible that other death effectors induced by P53 may be more sensitive to knockdown of Hdmx. Our initial examination of P53 target genes presented here supports the notion of differential gene activation following reduction of Hdmx. Ongoing microarray analyses will address this possibility. Alternatively, Hdmx may influence the kinetics at which cell cycle arrest or apoptotic responses are activated in response to Nutlin. Future experiments will address whether this could occur at the level of P53-dependent transactivation, or because Hdmx functionally interacts with effectors of arrest or apoptosis.

Is sensitization to Nutlin due solely to increases in P53-dependent transcription? Our data indicate that, when expressed in terms of absolute copy number (Fig. 7*B*), there are small increases in transactivation when Hdmx level is reduced. Thus, it is possible that sum of these changes from multiple P53 targets is sufficient to trigger cell death. If the transcription data is analyzed in terms of fold induction compared with untreated, we find that knockdown of Hdmx has no effect on Nutlin-induced, P53-dependent transactivation (data not shown). This is presumably because of the elevated level of transcription in Hdmx knockdown cells prior to Nutlin treatment. Therefore, we suggest that Hdmx could also act downstream of P53 target gene activation to modulate some P53-dependent processes. In this way, Hdmx may modulate P53 output at distinct nodes in the pathway. Interestingly, the majority of endogenous Hdmx is cytoplasmic in murine and human cells (58), whereas transcriptionally active P53 is by definition nuclear. Additionally, in human cancer cell lines that overexpress Hdmx, we find that a small proportion of total P53 is bound to Hdmx, consistent with their different subcellular localization (Fig. 1 and data not shown). How can the subcellular localization data be reconciled with the role of Hdmx as a transcriptional antagonist of P53? Perhaps the amount of nuclear Hdmx is sufficient for inhibition of P53 in unstressed cells, when P53 levels are very low. It is also possible that the cytoplasmic pool of Hdmx provides a "reservoir" to inactivate nuclear P53 during a stress response. Alternatively, the cytoplasmic pool of Hdmx may perform a role distinct from that of nuclear Hdmx. Although cytoplasmic binding partners for Hdmx have been reported, including the 14-3-3 family of proteins, the consequence of these interactions for cell survival remains unclear (51, 58, 59).

In this study, a combination of Hdmx knockdown and antagonism of Hdm2 was more effective than either treatment alone. Therefore, future strategies to design compounds that specifically inhibit Hdmx may be useful for combination chemotherapy with Nutlin-like compounds. Additionally, elucidation of factors that govern Hdmx stability may provide rational targets for additional drug development.

[8] M. Wade, unpublished observations.

REFERENCES

1. Hoh, J., Jin, S., Parrado, T., Edington, J., Levine, A. J., and Ott, J. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 8467–8472

2. Vogelstein, B., Lane, D., and Levine, A. J. (2000) *Nature* **408,** 307–310
3. Smith, N. D., Rubenstein, J. N., Eggener, S. E., and Kozlowski, J. M. (2003) *J Urol.* **169,** 1219–1228
4. Kmet, L. M., Cook, L. S., and Magliocco, A. M. (2003) *Cancer* **97,** 389–404
5. Ichikawa, A., Hotta, T., and Saito, H. (1993) *Leuk Lymphoma.* **11,** 21–25
6. Montes de Oca Lunaqq, R., Wagner, D. S., and Lozano, G. (1995) *Nature* **378,** 203–206
7. Jones, S. N., Roe, A. E., Donehower, L. A., and Bradley, A. (1995) *Nature* **378,** 206–208
8. Momand, J., Zambetti, G. P., Olson, D. C., George, D., and Levine, A. J. (1992) *Cell* **69,** 1237–1245
9. Kubbutat, M. H., Jones, S. N., and Vousden, K. H. (1997) *Nature* **387,** 299–303
10. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997) *Nature* **387,** 296–299
11. Krummel, K. A., Lee, C. J., Toledo, F., and Wahl, G. M. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 10188–10193
12. Parant, J., Chavez-Reyes, A., Little, N. A., Yan, W., Reinke, V., Jochemsen, A. G., and Lozano, G. (2001) *Nat. Genet.* **29,** 92–95
13. Sharp, D. A., Kratowicz, S. A., Sank, M. J., and George, D. L. (1999) *J. Biol. Chem.* **274,** 38189–38196
14. Shvarts, A., Steegenga, W. T., Riteco, N., van Laar, T., Dekker, P., Bazuine, M., van Ham, R. C., van der Houven van Oordt, W., Hateboer, G., van der Eb, A. J., and Jochemsen, A. G. (1996) *EMBO J.* **15,** 5349–5357
15. Stad, R., Ramos, Y. F., Little, N., Grivell, S., Attema, J., van Der Ebqq, A. J., and Jochemsen, A. G. (2000) *J. Biol. Chem.* **275,** 28039–28044
16. Francoz, S., Froment, P., Bogaerts, S., De Clercq, S., Maetens, M., Doumont, G., Bellefroid, E., and Marine, J. C. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 3232–3237
17. Toledo, F., Krummel, K. A., Lee, C. J., Liu, C. W., Rodewald, L. W., Tang, M., and Wahl, G. M. (2006) *Cancer Cell* **9,** 273–285
18. Gu, J., Kawai, H., Nie, L., Kitao, H., Wiederschain, D., Jochemsen, A. G., Parant, J., Lozano, G., and Yuan, Z. M. (2002) *J. Biol. Chem.* **277,** 19251–19254
19. Shvarts, A., Bazuine, M., Dekker, P., Ramos, Y. F., Steegenga, W. T., Merckx, G., van Ham, R. C., van der Houven van Oordt, W., van der Eb, A. J., and Jochemsen, A. G. (1997) *Genomics* **43,** 34–42
20. Lavin, M. F., Birrell, G., Chen, P., Kozlov, S., Scott, S., and Gueven, N. (2005) *Mutat. Res.* **569,** 123–132
21. Stommel, J. M., and Wahl, G. M. (2004) *EMBO J.* **23,** 1547–1556
22. Meulmeester, E., Maurice, M. M., Boutell, C., Teunisse, A. F., Ovaa, H., Abraham, T. E., Dirks, R. W., and Jochemsen, A. G. (2005) *Mol. Cell* **18,** 565–576
23. Pereg, Y., Shkedy, D., de Graaf, P., Meulmeester, E., Edelson-Averbukh, M., Salek, M., Biton, S., Teunisse, A. F., Lehmann, W. D., Jochemsen, A. G., and Shiloh, Y. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 5056–5061
24. Schwartzberg, L. (2006) *J. Support. Oncol.* **42,** Suppl. 1, 3–8
25. Vega-Stromberg, T. (2003) *J. Infus. Nurs.* **26,** 353–361
26. Vassilev, L. T., Vu, B. T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C., Fotouhi, N., and Liu, E. A. (2004) *Science* **303,** 844–848
27. Thompson, T., Tovar, C., Yang, H., Carvajal, D., Vu, B. T., Xu, Q., Wahl, G. M., Heimbrook, D. C., and Vassilev, L. T. (2004) *J. Biol. Chem.* **279,** 53015–53022
28. Tovar, C., Rosinski, J., Filipovic, Z., Higgins, B., Kolinsky, K., Hilton, H., Zhao, X., Vu, B. T., Qing, W., Packman, K., Myklebost, O., Heimbrook, D. C., and Vassilev, L. T. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 1888–1893
29. Kojima, K., Konopleva, M., Samudio, I. J., Shikami, M., Cabreira-Hansen, M., McQueen, T., Ruvolo, V., Tsao, T., Zeng, Z., Vassilev, L. T., and Andreeff, M. (2005) *Blood* **106,** 3150–3159
30. Secchiero, P., Barbarotto, E., Tiribelli, M., Zerbinati, C., di Iasio, M. G., Gonelli, A., Cavazzini, F., Campioni, D., Fanin, R., Cuneo, A., and Zauli, G. (2006) *Blood* **107,** 4122–4129
31. Stuhmer, T., Chatterjee, M., Hildebrandt, M., Herrmann, P., Gollasch, H., Gerecke, C., Theurich, S., Cigliano, L., Manz, R. A., Daniel, P. T., Bommert, K., Vassilev, L. T., and Bargou, R. C. (2005) *Blood* **106,** 3609–3617
32. Brummelkamp, T. R., Fabius, A. W., Mullenders, J., Madiredjo, M., Velds, A., Kerkhoven, R. M., Bernards, R., and Beijersbergen, R. L. (2006) *Nat. Chem. Biol.* **2,** 202–206
33. Coll-Mulet, L., Iglesias-Serret, D., Santidrian, A. F., Cosialls, A. M., de Frias, M., Castano, E., Campas, C., Barragan, M., de Sevilla, A. F., Domingo, A., Vassilev, L. T., Pons, G., and Gil, J. (2006) *Blood* **107,** 4109–4114
34. Follenzi, A., Ailles, L. E., Bakovic, S., Geuna, M., and Naldini, L. (2000) *Nat. Genet.* **25,** 217–222
35. Bottger, V., Bottger, A., Garcia-Echeverria, C., Ramos, Y. F., van der Eb, A. J., Jochemsen, A. G., and Lane, D. P. (1999) *Oncogene* **18,** 189–199
36. Marine, J. C., and Jochemsen, A. G. (2005) *Biochem. Biophys. Res. Commun.* **331,** 750–760
37. Kawai, H., Wiederschain, D., Kitao, H., Stuart, J., Tsai, K. K., and Yuan, Z. M. (2003) *J. Biol. Chem.* **278,** 45946–45953
38. Finch, R. A., Donoviel, D. B., Potter, D., Shi, M., Fan, A., Freed, D. D., Wang, C. Y., Zambrowicz, B. P., Ramirez-Solis, R., Sands, A. T., and Zhang, N. (2002) *Cancer Res.* **62,** 3221–3225
39. Migliorini, D., Denchi, E. L., Danovi, D., Jochemsen, A., Capillo, M., Gobbi, A., Helin, K., Pelicci, P. G., and Marine, J. C. (2002) *Mol. Cell Biol.* **22,** 5527–5538
40. Gentiletti, F., Mancini, F., D'Angelo, M., Sacchi, A., Pontecorvi, A., Jochemsen, A. G., and Moretti, F. (2002) *Oncogene* **21,** 867–877
41. Janicke, R. U., Sprengart, M. L., Wati, M. R., and Porter, A. G. (1998) *J. Biol. Chem.* **273,** 9357–9360
42. Crighton, D., Wilkinson, S., O'Prey, J., Syed, N., Smith, P., Harrison, P. R., Gasco, M., Garrone, O., Crook, T., and Ryan, K. M. (2006) *Cell* **126,** 121–134
43. Bensaad, K., Tsuruta, A., Selak, M. A., Vidal, M. N., Nakano, K., Bartrons, R., Gottlieb, E., and Vousden, K. H. (2006) *Cell* **126,** 107–120
44. Buolamwini, J. K., Addo, J., Kamath, S., Patil, S., Mason, D., and Ores, M. (2005) *Curr. Cancer Drug Targets* **5,** 57–68
45. Patton, J. T., Mayo, L. D., Singhi, A. D., Gudkov, A. V., Stark, G. R., and Jackson, M. W. (2006) *Cancer Res.* **66,** 3169–3176
46. de Toledo, S. M., Azzam, E. I., Dahlberg, W. K., Gooding, T. B., and Little, J. B. (2000) *Oncogene* **19,** 6185–6193
47. Khosravi, R., Maya, R., Gottlieb, T., Oren, M., Shiloh, Y., and Shkedy, D. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 14973–14977
48. Chen, L., Gilkes, D. M., Pan, Y., Lane, W. S., and Chen, J. (2005) *EMBO J.* **24,** 3411–3422
49. Wahl, G. M., Stommel, J. M., Krummel, K. A., and Wade, M. (2005) in *25 Years of p53 Research* (Wiman, K., and Hainaut, P., eds) pp. 73–113, Springer, Netherlands
50. Wahl, G. M. (2006) *Cell Death Differ.* **13,** 973–983
51. LeBron, C., Chen, L., Gilkes, D. M., and Chen, J. (2006) *EMBO J.* **25,** 1196–1206
52. Wendt, J., Radetzki, S., von Haefen, C., Hemmati, P. G., Guner, D., Schulze-Osthoff, K., Dorken, B., and Daniel, P. T. (2006) *Oncogene* **25,** 972–980
53. Zhang, Z., Wang, H., Li, M., Agrawal, S., Chen, X., and Zhang, R. (2004) *J. Biol. Chem.* **279,** 16000–16006
54. Uchida, C., Miwa, S., Kitagawa, K., Hattori, T., Isobe, T., Otani, S., Oda, T., Sugimura, H., Kamijo, T., Ookawa, K., Yasuda, H., and Kitagawa, M. (2005) *EMBO J.* **24,** 160–169
55. Legube, G., Linares, L. K., Lemercier, C., Scheffner, M., Khochbin, S., and Trouche, D. (2002) *EMBO J.* **21,** 1704–1712
56. Tian, L. F., Li, H. Y., Jin, B. F., Pan, X., Man, J. H., Zhang, P. J., Li, W. H., Liang, B., Liu, H., Zhao, J., Gong, W. L., Zhou, T., and Zhang, X. M. (2006) *Biochem. Biophys. Res. Commun.* **2,** 2
57. Xiong, S., Van Pelt, C. S., Elizondo-Fraire, A. C., Liu, G., and Lozano, G. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 3226–3231
58. Jin, Y., Dai, M. S., Lu, S. Z., Xu, Y., Luo, Z., Zhao, Y., and Lu, H. (2006) *EMBO J.* **25,** 1207–1218
59. Okamoto, K., Kashima, K., Pereg, Y., Ishida, M., Yamazaki, S., Nota, A., Teunisse, A., Migliorini, D., Kitabayashi, I., Marine, J. C., Prives, C., Shiloh, Y., Jochemsen, A. G., and Taya, Y. (2005) *Mol. Cell. Biol.* **25,** 9608–9620