Levels of HdmX Expression Dictate the Sensitivity of Normal and Transformed Cells to Nutlin-3

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Abstract
Hdm2 and HdmX coordinately regulate the stability and function of p53. Each is overexpressed in subsets of many different types of malignancy, and most of these subsets maintain wild-type p53. Nutlins, newly discovered small-molecule inhibitors of the Hdm2-p53 interaction, offer a novel strategy for therapy of tumors with wild-type p53. We now show that Nutlin-3 efficiently induces apoptosis and diminishes long-term survival of human fibroblasts transformed in vitro by Hdm2 but not HdmX. The resistance of cells overexpressing HdmX to Nutlin-3 is due to its inability to disrupt the p53-HdmX interaction, resulting in continued suppression of p53 activity. Although HdmX overexpression yielded cells resistant to Nutlin-3, ablation of HdmX expression by short hairpin RNA sensitized tumor cells to Nutlin-3-mediated cell death or arrest. Furthermore, deletion of the COOH-terminal RING finger domain of HdmX completely reversed the resistance to Nutlin-3, probably reflecting the requirement of the RING finger for interaction with Hdm2. Thus, the relative abundance of Hdm2 and HdmX and the specificity of Nutlin-3 for Hdm2 influence the sensitivity of cells to p53-dependent apoptosis or arrest in response to Nutlin-3. Our findings establish Hdm2 and HdmX as independent therapeutic targets with respect to reactivating wild-type p53 as a means for cancer therapy. (Cancer Res 2006; 66(6): 3169-76)

Introduction
Activation of p53 in response to various intracellular and extracellular stress signals leads to the transcriptional regulation of an ever increasing number of genes important for suppressing tumorigenesis (1). p53 can also induce transcription-independent apoptosis via direct interaction with Bcl family members that induce mitochondrial depolarization (2–5). p53 activity is suppressed in normal cells by Hdm2, a negative regulator that disrupts the interaction between p53 and the transcriptional machinery and promotes the nuclear export and ubiquitination of p53, ultimately leading to its proteosome-mediated degradation (6). The importance of Mdm2, the mouse isoform of Hdm2, in regulating p53 is shown by the embryonic lethality of mdm2 deletion (7, 8), which is specific for the loss of Mdm2-mediated p53 inhibition as deletion of both p53 and mdm2 results in viable animals that have an increased frequency of tumors similar to those with p53 deletion alone (9). MdmX, a structural homologue of Mdm2, binds to p53 and prevents p53-dependent transcription but has minimal E3 ubiquitin ligase activity and is unable to degrade p53 efficiently (10–14). The phenotype of the mdmX knock-out mouse is similar to that of the mdm2 knock-out (i.e., embryonic lethality that can be rescued by deletion of p53; refs. 15–17).

The relationship among p53, Hdm2, and HdmX is complex, with an interaction between Hdm2 and HdmX required for complete p53 regulation (11, 12, 18, 19). Hdm2 requires Hdm2 to move from the cytoplasm to the nucleus, whereas HdmX is required to extend the half-life of Hdm2, although it is less clear whether the Hdm2-HdmX complex regulates p53 directly (18). Furthermore, the hdm2 gene is transcriptionally regulated by p53 (20, 21). Therefore, activation of p53 induces hdm2 transcription, and the increased levels of Hdm2 protein down-regulates p53 and HdmX. In response to DNA damage, the Hdm2-p53 and HdmX-p53 interactions are disrupted, facilitating the tumor suppressor functions of p53 (22, 23). Conversely, DNA damage enhances the degradation of HdmX and Hdm2 via ATM-dependent phosphorylation, leading to impaired deubiquitination of both proteins by the HAUSP deubiquitinating enzyme (24, 25).

Although the two proteins differ with respect to their ability to ubiquitinate p53, the p53-binding pockets of Hdm2 and HdmX are similar. Not surprisingly, both proteins are found overexpressed in human tumors, including breast carcinomas and sarcomas, many of which maintain wild-type p53 (26–28). Therefore, the selective pressure to eliminate p53 commonly results in either inhibition of wild-type p53 activities or p53 mutation. Tumors harboring both classes of p53 inactivating events have been the focus of studies to identify novel anticancer strategies. Diverse approaches aimed at disrupting the interaction between Hdm2 and p53 to facilitate wild-type p53 activities include the use of antibodies directed at Hdm2, inhibitory peptides, and antisense oligonucleotides or small interfering RNAs to inhibit Hdm2 expression (29–31). Recently, two drug discovery screens identified a series of Nutlin compounds and RITA, low molecular weight compounds capable of disrupting the p53-Hdm2 interaction, leading to p53-dependent toxicity in tumor cell lines and xenograft tumors in mice (32, 33). In addition to activating wild-type p53, the PRIMA-1 compound was also found to structurally alter tumor-derived p53 mutants and restore their wild-type conformation, inducing apoptosis in a series of cancer cell lines (34).

Recent identification of genetic elements capable of cooperating to transform human cells has permitted scientists to examine the contribution of specific genes to the transformation process. Evaluation of the response to therapeutic agents of cells transformed in vitro may also provide conclusive evidence regarding the involvement of a specific gene in resistance to a given treatment. In this report, we expand upon a recent model of human cell transformation that is dependent upon p53 inactivation, asking whether the genetic event responsible for p53 inactivation dictates...
the response to Nutlin-3. Our model system consists of normal human fibroblasts transduced with adenoviral E1A, oncogenic Ras mutant (RasV12), and the hTERT subunit of telomerase. When cells carrying these genes lose p53 function in response to Mdm2, Hdm2, HdmX, or knock down by short hairpin RNA (shRNA), they become fully transformed (35). We report here that the inactivation of p53 by Hdm2 or HdmX yields cells that differ greatly in their sensitivity to Nutlin-3 due to the selective disruption of p53-Hdm2 interactions. Cells transformed by HdmX have a significant survival advantage, whereas ablation of HdmX expression using shRNA sensitizes cells to Nutlin-3. Our findings indicate that the interactions among p53, Hdm2, and HdmX must be maintained in proper balance for appropriate tumor surveillance and suppression. Transformation due to Hdm2 or HdmX overexpression alters the balance of this sensitive regulatory network, with profound implications for the therapeutic efficiency of specific compounds.

Materials and Methods

Cell lines and culture conditions. IMR90 and HCT116 cells were grown in a humidified atmosphere containing 5% CO2 in DMEM (Life Technologies, Grand Island, NY) supplemented with antibiotics and 10% fetal bovine serum (U.S. Biochemical Corp., Cleveland, OH). hTERT-HME1 cells were purchased from Clontech (Mountain View, CA) and grown in a humidified atmosphere containing 5% CO2 in Medium 171 with mammary epithelial growth supplement (Cascade Biologies, Portland, OR). Nutlin-3 was purchased from Cayman Chemical (Ann Arbor, MI). Cells were plated 24 hours before Nutlin-3 was added to fresh media at the indicated concentrations. For survival and growth assays, medium containing Nutlin-3 was removed after 24 hours for IMR-ERT cells or 72 hours for hTERT-HME1, replaced with fresh medium, and cells were grown an additional 5 days to 2 weeks before staining with methylene blue in 50% methanol. The stain was quantified following extraction with 0.5 mol/L HCl by reading the absorbance at 595 nm.

Plasmids and retroviral infection. Plasmids (pBabe-Bleo H-Ras-V12, pLPCX-E1A, 1V-Hdm2, and shRNA constructs) and protocols for the production and growth of IMR-ERT cells and derivatives have been described elsewhere (36, 37). HdmX cDNA was kindly provided by Dr. Steven Berberich (Wright State University, Dayton, OH). The FLAG-tagged versions of HdmX were created by PCR amplification using PFU turbo polymerase (La Jolla, CA), the forward primer containing a HindIII site, GGGGAAGCTTTGACATCATTTTCCACCTCTGCT and reverse primer, ACCAATATCTCCAAAAATATCTGATTATTCCTAAGGGGG. To create the full-length HdmX expression vector, the PCR product was cut with HindIII alone and cloned into the pFLAG-CMV2 vector (Sigma, St. Louis, MO) cut with HindIII and EcoRI. To create the HdmXΔC expression vector, the PCR product was cut with HindIII and EcoRI. An EcoRI site within the HdmX cDNA 1177-1182 removes amino acids 395 to 490 and, upon ligation into the HindIII/EcoRI sites of the pFLAG-CMV2 vector, adds 11 unique amino acids to the end of the protein. Each pFLAG-CMV2 vector was subsequently cut with Sau3AII [unique to the cytomegalovirus (CMV) promoter] and BamHI (3’ MCS) and cloned into the retroviral vectors pLNCX2 and pLCX (Clontech). Retroviral encoding the cDNA of interest were packaged in Phoenix-Ampho (pBabe, pLPCX, and pLNCX2 derivatives) or 293T cells (LV-Hdm2, sh-Scrambled, or sh-p53) together with a second-generation packaging construct (pcMV-dR8.74) and pMD2G (both provided by D. Trono, University of Geneva). Supernatant media containing virus were grown an additional 5 days to 2 weeks before a 0.22-μm filter, and added to cells overnight. Uninfected cells were observed by microscopy. A, B, and C, cells described above were observed by microscopy 96 hours after Nutlin-3 treatment, and colonies were counted.

Soft agar assays. Cells (1 × 103 per 60-mm dish) were resuspended in 0.6% Type VII agarose (Sigma) and plated onto a bottom layer of 1.2% agar. For Nutlin-3 treatment, cells were plated into soft agar and left to grow for 24 to 48 hours, after which 1 to 2 mL of fresh media with or without 10 μmol/L Nutlin-3 was added to the topagar and left 24 hours to 2 weeks before photographing and quantifying.

Western immunoprecipitation. Whole-cell extracts and immunoprecipitation were carried out as previously described with exceptions as noted (11). Hdm2 and HdmX were immunoprecipitated using 2A10 (Oncogene Research Products, La Jolla, CA) and BL1258 (Bethyl Laboratories, Montgomery, TX) antibodies, respectively. Extracts containing equal quantities of proteins, determined by the Bradford method, were separated by SDS-PAGE (8-12.5% acrylamide) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Antibodies to p53 (DO-1 and C19 goat polyclonal) and p21Waf1 (C19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Additional Hdm2 antibodies IF2 and 4B11 were from Oncogene Research Products. Primary antibodies were detected with goat-anti-mouse or goat-anti-rabbit antibody conjugated to horseradish peroxidase (Hoffman-La Roche, Basel, Switzerland), using enhanced chemiluminescence (Perkin-Elmer, Wellesley, MA). For in vitro binding assays, GST-HdmX was produced in bacteria from the pGEX4 vector. Recombinant Hdm2 and p53 protein were produced and ELISA-based interaction studies were done as previously described (38). Briefly, recombinant p53 was bound to an ELISA plate (96-well format), and recombinant Hdm2 or HdmX were preincubated with the indicated doses of Nutlin3, added to the
well containing p53, and incubated for 1 hour. After extensive washing, the amount of Hdm2 or HdmX bound to p53 was quantified using Hdm2- or HdmX-specific proteins via colorimetric analysis.

RNA isolation and reverse transcription-PCR. Total RNA was isolated using Qiagen RNeasy miniprep kit (Qiagen, Valencia CA). RNA was analyzed for Noxa and p53 expression using an Applied Biosystems GeneAMP PCR System 9700 and Applied Biosystems assays Hs00382168_m1 and Hs00153340_m1 at the Gene Expression Array Core Facility of the Comprehensive Cancer Center of Case Western Reserve University and University Hospitals of Cleveland. Relative Noxa expression was normalized to p53 expression and plotted in Fig. 1D.

Results

Fibroblasts transformed by HdmX in vitro are resistant to Nutlin-3. To determine whether the genetic event responsible for wild-type p53 inactivation dictates the response of cells transformed in vitro to Nutlin-3, IMR90 fibroblasts expressing E1A, RasV12, and hTERT (termed IMR-ERT cells) were infected with amphotropic retroviruses or lentiviruses capable of expressing Hdm2 or HdmX, shRNA to knockdown p53 expression (sh-p53) or control vectors (empty vector, green fluorescent protein, and a scrambled shRNA) as described elsewhere (36). The populations of cells were plated into soft agar and permitted to grow for 48 hours before treatment with 10 μmol/L Nutlin-3. Within 24 hours of Nutlin-3 addition, the few colonies forming in the vector-infected cells and most of the colonies expressing Hdm2 showed signs of disorganization and blebbing uncharacteristic of normal colonies grown in soft agar (Fig. 1A). These colonies also showed little sign of additional growth following Nutlin-3 addition (Fig. 1B, left; data not shown). In contrast, cells infected with sh-p53 or HdmX showed little disorganization and continued to grow throughout the experiment (Fig. 1A and B). Quantification of soft agar growth showed a 75% reduction in colonies from cells overexpressing Hdm2 compared with no significant decrease in colony formation in cells expressing HdmX or shRNA to p53 (Fig. 1C).

To examine further the mechanism of resistance observed in the HdmX and sh-p53 cells following Nutlin-3 treatment, we plated cells onto tissue culture dishes and treated them with increasing doses of Nutlin-3. After 24 hours of treatment, cells were analyzed by fluorescence-activated cell sorting (FACS) and the Western method or fed with fresh media to monitor their long-term survival. In agreement with the observations made with cells in soft agar, vector controls and Hdm2-expressing cells showed a significant increase in the proportion of cells with a sub-G1 DNA content, indicating that the cells were undergoing apoptosis (Fig. 2A, top). In contrast, HdmX-expressing and sh-p53 cells showed no little sign of undergoing apoptosis following Nutlin-3 treatment (Fig. 2A).

This observation is consistent with the differential survival of each population when long-term survival was measured. Following treatment with 10 μmol/L Nutlin-3, relative survival was 5% for the vector-infected control and 14% for the Hdm2-expressing cells. In contrast, 74% of the HdmX-expressing cells and 100% of sh-p53 expressing cells survived treatment with 10 μmol/L Nutlin-3, again indicating that HdmX-mediated p53 inhibition is refractory to Nutlin-3 (Fig. 2B). These observations were made with two
independent pools of Hdm2-expressing and HdmX-expressing cells and with cells generated from each control vector (including a nonspecific scrambled shRNA; data not shown). Western analysis of the vector and sh-p53 cells showed that Hdm2 and p21 induction in response to Nutlin-3 treatment was p53 dependent, and also that the level of HdmX decreased in a p53-dependent, and presumably Hdm2-dependent, manner (Fig. 2C). A similar decrease in endogenous HdmX has been observed in normal human mammary epithelial cells and HCT116 colorectal cancer cells (data not shown). Given the resistance to Nutlin-3 conferred by HdmX overexpression, the down-regulation of HdmX probably contributes to the full response of the vector-infected and Hdm2-expressing cells. Comparison of Noxa expression by real-time PCR showed that HdmX overexpression confers resistance to the Nutlin-3-mediated p53 activation of Noxa, whereas Hdm2-overexpressing cells showed significant Noxa induction (Fig. 2D).

Nutlin-3 specifically disrupts the interaction between Hdm2 and p53. One explanation for the resistance of HdmX-expressing cells to Nutlin-3 treatment is that, in contrast to its ability to prevent Hdm2-p53 complexes (33), Nutlin-3 is unable to disrupt HdmX-p53 complexes. To test this point, IMR-ERT cells expressing Hdm2 and HdmX were treated with 10 μmol/L Nutlin-3 for 24 hours, after which Hdm2 or HdmX were immunoprecipitated, and their interactions with p53 were examined by Western analysis. After Nutlin-3 treatment, there was far less p53 bound to Hdm2 (Fig. 3A, compare lanes 1 and 2), although there was significantly more Hdm2 immunoprecipitated (compare lanes 1 and 2) and significantly more p53 present in the total lysate (compare lanes 5 and 6). The converse is true in HdmX-expressing cells, where far less HdmX was immunoprecipitated from the extract (compare lanes 3 and 4), yet the amount of p53 coprecipitated was greater (compare lanes 1 and 2, p53 blot). Furthermore, when p53 ubiquitination was examined in a longer exposure, it was noted that HdmX binds efficiently even to ubiquitinated p53 (Fig. 3A, bottom). We also observed an increase in HdmX binding to p53 upon immunoprecipitation of p53 from both Hdm2- and HdmX-expressing cells (data not shown). Further analysis of the p53-Hdm2 and p53-HdmX interactions using bacterially produced recombinant p53, Hdm2, and HdmX showed that Nutlin-3 directly disrupted the interaction between Hdm2 and p53, while doing little to disrupt the direct interaction between HdmX and p53 (Fig. 3B).

COOH-terminal RING finger domain is required for HdmX-mediated Nutlin-3 resistance. Because there is some debate regarding whether Hdm2 and HdmX require one another for full functionality, we next tested whether the interaction between Hdm2 and HdmX was required for the p53 regulation observed (11, 12, 39, 40). The COOH-terminal RING domain of HdmX, necessary for the interaction between Hdm2 and HdmX, was deleted and IMR-ERT cells were created that express the HdmXΔC mutant. Consistent with our initial observations, vector control cells showed an increasing population of cells having sub-G1 DNA content and significantly reduced long-term survival, whereas HdmX-expressing cells showed little sign of undergoing apoptosis and little decrease in long-term survival (Fig. 4A and B). Examination of the HdmXΔC cells revealed a sensitivity to Nutlin-3 similar to vector control cells in both short-term survival, as shown by FACS, and long-term survival.

Western analysis of each of the populations illustrated that p53 was induced by Nutlin-3 in HdmX-expressing cells but was impaired in its ability to induce p21 and Hdm2. In contrast, HdmXΔC-expressing cells show similar levels of Hdm2 and p21 induction relative to the control cells, again confirming the need for the COOH-terminal RING finger domain of HdmX for full p53 inhibition (Fig. 4C). Of interest, HdmX protein levels continued to decrease following Nutlin-3 treatment, whereas the HdmX mutant lacking the RING finger domain was not destabilized. To test whether p53 is degraded in cells expressing HdmX, cells were treated with Nutlin-3 for 24 hours and then treated with the proteosome inhibitor ALLN for an additional 4 hours. Combined treatment with Nutlin-3 and proteosome inhibitor induced more p53 accumulation and more slowly migrating forms of p53, consistent with p53 ubiquitination and degradation (Fig. 4D).

HdmX levels determine the sensitivity of normal and cancer cells to Nutlin-3. To insure that the results observed with the IMR-ERT cells are not the result of an undefined effect of E1A or RasV12 expression, we confirmed our findings in hTERT-HME1 cells, an untransformed mammary epithelial cell line. This experiment is particularly important in light of the previously reported interaction between HdmX and E1A, although we were unable to detect E1A bound to either HdmX or Hdm2 in our cells (data not shown). In response to Nutlin-3 treatment, hTERT-HME1 cells undergo arrest rather than apoptosis (data not shown). Similar to our findings in IMR-ERT cells, vector-infected and HdmXΔC-expressing cells had a 94% decrease in cell number following 10 μmol/L Nutlin-3 treatment relative to untreated cells (Fig. 5A). In contrast, HdmX expression conferred significant protection from the
Nutlin-3–mediated growth inhibition, with only a 32% decrease in cell number relative to control at the same concentration (Fig. 5A). Also consistent with the IRM-ERT cells is the decreased induction of Hdm2 and decreased expression of exogenous HdmX expression following Nutlin-3 treatment. The vector-infected and HdmXΔC-expressing cells showed equivalent Hdm2 induction (Fig. 5B).

We also used shRNA directed against hdmX to determine whether cells having lower levels of HdmX would be more sensitive to Nutlin-3 treatment. HCT116 colorectal cancer cells were infected with lentiviral sh-HdmX or sh-Hdm2 constructs as previously reported (37), and it was verified by Western analysis that the expression of each protein was inhibited (Fig. 5C). When the HCT116 derivatives were plated into soft agar to examine their ability to grow anchorage independently, all three cell lines grew with similar efficiencies. Following Nutlin-3 treatment, the growth of cells expressing sh-SCR (scrambled shRNA) and sh-Hdm2 was compromised, although colonies did develop over the course of the experiment. In contrast, there was very little growth of cells expressing sh-HdmX (Fig. 5D). Although these data are qualitative, they are representative of numerous plates and experiments.

Thus, in three different cell models, we conclude that HdmX expression is a determining factor of Nutlin-3 sensitivity. HdmX expression inhibits Hdm2 induction by decreasing Hdm2 transcription (a global effect of HdmX-mediated p53 inhibition) and by active cooperative degradation of Hdm2-HdmX complexes. With decreased levels of Hdm2 present, the stable HdmX protein continues to repress p53-dependent apoptosis or cell cycle arrest.

Combining Nutlin-3 and Adriamycin leads to the synergistic killing of Nutlin-3–resistant cells. Although the lack of p53-dependent apoptosis or arrest in cells expressing HdmX is clear, the fact that p53 protein is still stabilized in response to Nutlin-3 treatment may be exploited if the HdmX-mediated inhibition of p53 can be prevented. Importantly, much like Hdm2-p53 complexes, HdmX-p53 complexes can be disrupted by phosphorylation in response to DNA damage (22, 23). To assess whether DNA damage can facilitate the activity of p53 in HdmX-expressing cells treated with Nutlin-3, we first treated HdmX- or Hdm2-expressing cells with a low dose of Nutlin-3 (3 μmol/L), and 24 hours later, added Adriamycin, at either 10 or 20 nmol/L, for an additional 24 hours. Nutlin-3 treatment alone reduced cell survival by 20% in HdmX-expressing cells and 50% in Hdm2-expressing cells. Similarly, 10 nmol/L Adriamycin alone modestly decreased survival of both populations by 20%. However, combined treatment with Nutlin-3 and Adriamycin synergistically reduced the viability of both populations achieving a 98% reduction in viability for both populations at the highest dose of Adriamycin. Western analysis revealed that the induction of Hdm2 protein correlated inversely with survival, whereas HdmX protein levels correlated directly with survival, arguing that HdmX protein levels correlated directly with survival, suggesting that HdmX degradation is an important factor in the full activation of p53 in response to nongenotoxic induction.

Discussion
The discovery of compounds that can reactivate p53 opens the door to genotype-based treatment: knowing the reason for p53 inactivation in a given tumor may well dictate the choice of therapy. To understand the responses of tumors with a given genotype to a specific treatment, biomedical researchers have commonly used a limited set of genetically undefined tumor cell lines, using a correlative analysis of mechanism to indicate potential therapeutic approaches. The recent identification of small sets of cellular and viral proteins that can cooperate to transform normal human cells permits a more precise analysis of the involvement of specific proteins in resistance or sensitivity to a given treatment.  

p53-Hdm2-HdmX triad: balance is the key. In normal cells, a defined balance in the relative levels of Hdm2 and HdmX, either
individually or cooperatively, regulate p53 functions, keeping target gene transcription at levels that do not cause apoptosis or cell cycle arrest. Because hdm2 is a transcriptional target of p53, its expression also remains low relative to the levels induced after p53 activation, whereas hdmX expression remains constitutive in all experimental conditions examined thus far.

In this report, we use isogenic populations of normal human fibroblasts transformed by hTERT, E1A, oncogenic Ras, and either Hdm2, HdmX, or shRNA to p53 to determine the effects of Nutlin-3 in cells with wild-type p53. Hdm2-expressing cells are sensitive to Nutlin-3, whereas HdmX-expressing cells or cells with low levels of p53 remain unaffected. Upon treatment with Nutlin-3, Hdm2-p53 complexes are disrupted, leading to stabilization of p53. Provided that the level of HdmX protein is not high, the disruption of Hdm2-p53 complexes leaves enough free p53 to transactivate proapoptotic and/or cell cycle arrest genes, as well as hdm2 itself. The additional Hdm2 protein binds to HdmX, promoting its degradation and leading to a positive feedback loop, in which p53 activity continues to increase.

Unbalanced HdmX levels dictate sensitivity and resistance to Nutlin-3. In cells overexpressing HdmX, the level of Hdm2 is low due to the HdmX-mediated inhibition of p53-dependent transactivation. Upon treatment with Nutlin-3, HdmX-p53 complexes are unaffected, and the increase in p53 levels comes from the smaller pool of Hdm2-p53 complexes, leading to diminished induction of p53 target genes, including hdm2. Lower levels of Hdm2 lead to decreased HdmX degradation and ultimately more HdmX-p53 complexes, which keep p53 inactive. In contrast, decreased HdmX expression, as seen in our experiments using shRNA to HdmX, has the opposite effect. In this instance, p53-Hdm2 interactions are predominant, leaving the majority of p53 free following Nutlin-3 treatment. This effect translates into increased growth suppression or apoptosis, as seen in our experiments with cells growing in soft agar. What remains unclear from our experiments, and from most studies that examine Hdm2-HdmX interactions, is the contribution of the Hdm2-HdmX dimer to p53 regulation. Although Hdm2 and HdmX have been reported to require one another for stability and nuclear localization, respectively (18), whether a heterodimer can bind to p53 and ubiquitinate it remains unclear.

The fact that HdmX-p53 interactions are not disrupted by Nutlin-3 is, at first glance, unexpected. Detailed analysis of peptides by
phage display suggested a strong similarity between the p53 binding domains of HdmX and Hdm2. However, there were also clear differences in the ability of specific peptides to disrupt p53-Hdm2 and p53-HdmX interactions, with as much as a 10-fold difference in competition (41). This may reflect the differences noted between critical amino acids shown to be important point contacts between p53 and Hdm2: 4 of 14 differ between HdmX and Hdm2. Additionally, the p53-binding domains of the two proteins share only 66% similarity, more than enough difference to explain the differential effects of Nutlin-3 on Hdm2 and HdmX described here.

Is p53 function in tumor cells ever really wild type? The observations presented here are important in a number of respects. First, Hdm2 and HdmX overexpression are observed in diverse tumor types, predominantly in soft tissue and breast cancers. Conventional therapeutic approaches, such as chemotherapy and radiation therapy, tend to perform poorly in tumors that overexpress Hdm2, which is commonly associated with advanced, metastatic, and hormone-independent cancers. There is little information regarding whether HdmX-overexpressing tumors respond similarly. However, the cells transformed in vitro by Hdm2 or HdmX overexpression described here show similar apoptosis-resistant responses to Adriamycin or ionizing radiation (Fig. 6).1 Danovi et al. recently described the first comprehensive analysis of HdmX, Hdm2, and p53 expression in multiple tumor types (26). HdmX protein was overexpressed in nearly 20% of the breast, colon, and lung cancers examined. Sequence analysis of the p53 gene in tumors over-expressing HdmX revealed that p53 was wild type, and tumors having HdmX amplification had normal Hdm2 copy number (26). In addition, at least two different splice variants of HdmX have been detected in tumor samples, the expression of which correlates with decreased patient survival (42–44).

The use of Nutlin-3 has worked well in specific tumor cell lines and xenograft tumors, acute myeloid leukemias, and multiple myelomas (33, 45, 46). Molecules that bind to Hdm2 may work well in tumors overexpressing Hdm2, but the failure of Nutlin-3 to affect HdmX-p53 interactions raises an important consideration regarding the use of therapeutic approaches aimed at promoting the tumor-suppressive functions of wild-type p53. Caution aside, the discovery of low molecular weight compounds that can reactivate p53 function in any tumor is an important step in the right direction, and, surely, additional refining of these approaches will continue to uncover novel ways to induce p53, with the hope of selective tumor cell killing.

Acknowledgments

Received 10/24/2005; revised 1/3/2006; accepted 1/19/2006.

Grant support: Grants P30CA43703 (Flow Cytometry Core Facility and Gene Expression Array Core Facility of the Case Comprehensive Cancer Center), F32HL072661 (M.W. Jackson), RO1CA109262 (L.D. Mayo), and R01GM09345 (G.R. Stark).

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We thank Steven Berberich for the HdmX cDNA and Richard Igglo (Oncogene Group, Swiss Institute for Experimental Cancer Research [ISREC], Epalinges, Switzerland) for the sh-Hdm2 constructs.

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