Excess HDM2 Impacts Cell Cycle and Apoptosis and Has a Selective Effect on p53-dependent Transcription

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Shuichi Ohkubo1*, Tomoaki Tanaka1, Yoichi Taya1, Kenji Kitazato1, and Carol Prives1,†1

From the 1Department of Biological Sciences, Columbia University, New York, New York 10027, 6Cancer Research Laboratory, Taiho Pharmaceutical Co., Ltd., 1-27 Misugida, Hanno, Saitama 357-8527, Japan, and 6National Cancer Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

Mutational inactivation of p53 is only one of the ways that tumors lose p53 function. An alternate route is through overexpression of HDM2, the negative regulator of p53. To further understand how excess HDM2 regulates p53-mediated functions, we generated H1299 cell clones that constitutively express both ectopic HDM2 and tetracycline-regulated inducible p53. We found that over a range of p53 concentrations constitutively expressed HDM2 did not affect the levels of p53 protein. Nevertheless, cells with excess HDM2 displayed numerous changes in their response to p53. After DNA damage, such cells had both increased p53-mediated G2 arrest and reduced cell death. They also showed selective impairment of p53 target gene induction in that some p53 targets were unaffected whereas others were markedly less well induced in the presence of extra HDM2 protein. We also found that excess HDM2 was correlated with reduced p53 acetylation but did not affect p53 association with target promoters in vivo. Indeed, there was no significant difference in the amount of HDM2 associated with p53 at target promoters that differed in their expression depending on the presence of extra HDM2. Thus, HDM2 can selectively down-regulate the transcription function of p53 without either degrading p53 or affecting the interaction of p53 with target promoters.

The p53 tumor suppressor gene is most frequently mutated in human cancers (1), suggesting that p53 plays a critical role in the control of cell growth. p53 is a transcription factor that modulates the transcription of a large number of target genes in response to various cellular stresses such as DNA damage, hypoxia, heat shock, and viral infection (1–3). The activation of p53 leads to diverse cellular processes, including cell cycle regulation, apoptosis, senescence, and differentiation. The activity of p53 is regulated by multiple mechanisms. Post-translational modifications of p53 may change its half-life, intracellular localization, conformation, DNA binding, and interaction with regulatory proteins (1–6).

Mimetic double minute clone 2 (mdm2) was originally cloned as an amplified gene in a spontaneously transformed Balb/c cell line 3T3DM (7, 8). The human homologue of Mdm2 (HDM2) is also frequently amplified in some human cancers, especially in sarcomas and brain tumors (reviewed in Ref. 1). In such tumors p53 is often wild type, consistent with the supposition that HDM2 inactivation of p53 can at least partially phenocopy mutation of p53 itself. HDM2 regulates p53-mediated growth suppression and apoptosis by inhibiting p53 (9).

Because HDM2 is a transcriptional target of p53 (10, 11), HDM2 and p53 form a negative feedback loop in which p53 activates HDM2 which in turn down-regulates p53.

HDM2 can bind to the N-terminal transcriptional activation domain of p53 (12, 13) and modulates p53 function by several ways. First, HDM2 can block the transactivation ability of p53 (13–15). Although the interaction of HDM2 and p53 is needed for inhibition of p53-mediated transactivation, HDM2 itself possesses a domain that can directly repress basal transcription (15). Second, HDM2 functions as a ubiquitin-protein isopeptide ligase and facilitates degradation of p53 by the proteasome (16–19). The C terminus of HDM2 spans its RING domain, which is essential for HDM2 ubiquitin ligase activity toward p53 and itself (20, 21). Third, HDM2 plays a role in translocation of p53 from the nucleus to the cytoplasm (22, 23). This activity requires the nuclear export signal of p53, and ubiquitination of the C terminus of p53 by HDM2 contributes to the efficient export of p53 (24). In addition, HDM2 is a potential inhibitor of p53 acetylation (25–28) that occurs after some forms of cellular stress and that regulates the transactivation and stability of p53 (29).

Until recently most reports investigated the functional regulation of p53 by HDM2 by either transient overexpression or in vitro assays, although at least two reports have provided evidence that excess endogenously expressed HDM2 can dramatically affect p53 function. First, Knights et al. (30) characterized cells with or without amplified Mdm2 that were derived from carcinogen-treated murine epidermal 291 cells and identified deficiencies in p53 responses and activities in cells with excess Mdm2. Second, Bond et al. (31) discovered that a single nucleotide polymorphism in the human HDM2 promoter creates a strong Sp1-binding site and thereby results in increased HDM2 expression that is correlated with both defective p53 response in cell cultures and increased cancer susceptibility in the human population. Our goal was to investigate how excess HDM2 regulates the function of p53 in vivo by using isogenic stable H1299 cell lines that both contain tetracycline-regulated inducible p53 and constitutively express FLAG-tagged HDM2. By using this approach we can change the ratio of p53 and HDM2 protein and measure the function of p53 in the same genetic background. We observed that degradation of p53 is not the dominant mode by which HDM2 inhibits the function of p53 in these cells and that HDM2 can selectively down-regulate the ability of p53 to regulate expression of endogenous target genes.

MATERIALS AND METHODS

Plasmids—Human HDM2 containing two N-terminal FLAG epitope tags and a C-terminal HA epitope tag was generated and amplified by PCR (Expand High Fidelity PCR System, Roche Applied Science) and cloned onto pcDNA3 (Invitrogen) to generate pcDNA-F-HDM2. The human p53 expression plasmid was a gift from B. Vogelstein. The pTK-Hyg and pEGFP-F constructs were from Clontech.


**Cell Lines and Transfection**—The cell lines used in this study are all clonally derived from H1299 human non-small cell lung carcinoma cells. A cell line expressing tetracycline-regulated p53 (H24-14) was described previously (32). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone). H24-14 cells were cultured in the presence of puromycin (2 μg/ml; Sigma), G418 (400 μg/ml; Invitrogen), and tetracycline (4 μg/ml; Sigma). To establish cell lines that both contain inducible p53 and constitutively expressed HDM2, pcDNA/F-HDM2 and pTK-Hyg were transfected into H24-14 cultures using Lipofectamine (Invitrogen). Clones were isolated and expanded in the presence of 400 and 300 μg of hygromycin B (Invitrogen) per ml, respectively. Expression of FLAG-HDM2-HA in isolated clones was detected by immunoblotting analysis of the cell extracts. One of the clones that express FLAG-HDM2-HA at relatively high levels when compared with other clones was selected for the experiments and designated as H24-14/HDM2#1. Additional clones were also isolated and characterized.

To confirm characteristics of FLAG- and HA-tagged HDM2, H1299 cells were seeded 24 h before transfection on 10-cm dishes and then transfected with 5 μg of p53 expression plasmid with or without 5 μg of pcDNA/F-HDM2 using Lipofectamine. 24 h after transfection, the levels of p53 and FLAG-HDM2-HA were determined by immunoblotting. The binding ability of FLAG-HDM2-HA to p53 was determined by immunoprecipitation experiments.

**Flow Cytometry Analysis**—Both adherent and detached cells were collected and fixed with 5 ml of ice-cold methanol for 1 h at −20 °C. The fixed cells were suspended in PBS containing RNase A (50 μg/ml; Sigma) and propidium iodide (60 μg/ml; Sigma). The stained cells were analyzed in a fluorescence-activated cell sorter (FACS Calibur, BD Biosciences), and data were analyzed with ModFit LT software.

**Antibodies**—Monoclonal antibodies PAb1801 and DO-1 were used to detect p53, and monoclonal antibodies SMP-14 and 2A10 were used to detect HDM2. PIG3 antibody was a generous gift from D. Hill (Oncogene Research Products). The cyclin G1, polyclonal antibody (33), the anti-acetyl p53 antibody at lysine 382 (34), and the anti-phospho-p53 were purchased from Sigma, 5′-CTCTAAGGTGGCAGGTG-3′, GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FACS, fluorescence-activated cell sorter.

**Immunoblotting and Immunoprecipitation**—48 h after removal of tetracycline, cells were washed in PBS and lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, 10 mM NaF, 1 mM sodium orthovanadate, 5 μM trichostatin A, 1 mM phenylmethylsulfonyl fluoride, 3 μg/ml leupeptin, 3 μg/ml pepstatin A, and 10 μg/ml aprotinin. Lysates were then centrifuged at 13,000 rpm for 20 min, and the supernatants were collected. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell). Membranes were blocked with 5% nonfat dried milk in PBST (PBS, 0.05% Tween 20) and probed with appropriate antibodies.

For immunoprecipitation, PAb1801 was covalently attached to Protein A-Sepharose (1801 beads) (Amersham Biosciences). Cell lysates were incubated with 1801 beads or anti-FLAG M2-agarose affinity gel (Sigma) for 4 h at 4 °C, and the immunoprecipitates were then washed four times in lysis buffer, boiled in electrophoresis sample buffer for 5 min, and used for immunoblotting as described above.

**Immunostaining**—Cells were plated onto coverslips and cultured with or without tetracycline (4 μg/ml) for 48 h. Cells were fixed in 4% paraformaldehyde for 20 min and then incubated with 0.2% Triton X-100 for 5 min. After blocking, the coverslips were incubated with anti-p53 (FL393) and anti-HDM2 (SMP-14) followed by incubation with anti-rabbit Alexa Fluor 488 conjugate (Molecular Probes) and antirat Alexa Fluor 594 conjugate (Molecular Probes). Nuclei were visualized with 4,6-diamidino-2-phenylindole (Staining) staining.

**RT-PCR**—48 h after removal of tetracycline, total RNA was isolated by use of an RNeasy kit (Qiagen), and contaminating DNA was digested with 10 units of DNase I (Promega) at 37 °C for 30 min. The total RNA (5 μg) was reverse-transcribed using the ThermoScript RT-PCR system (Invitrogen), and 5% of the cDNA products was subjected to PCR using the Expand High Fidelity PCR system (Roche Applied Science) with five different pairs of primers as follows: pig3 oligonucleotides, 5′-CCGGGGGAGGTGGAAGTC-3′/5′-TCCAGCATCCGCGTCTTGAAC-3′; pig2 oligonucleotides, 5′-CCTCAAGGTGGCAGGTG-3′/5′-GAAAGAAGTTAGCTGGGGCTC-3′; bax oligonucleotides, 5′-GCGGACCGACGTAGAACC-3′/5′-ATCCACAGTCTCTGTTGGTG-3′. The cycling conditions were as follows: a denaturation step at 94 °C for 2 min followed by 20 cycles (for gapdh and pi21), or 25 cycles (for pig3, bax, and 14-3-3σ) at 94 °C for 30 s, 57 °C for 30 s and 72 °C for 2 min, as well as a final extension of 72 °C for 7 min. PCR products were loaded onto a 1.5% agarose gel and were visualized with ethidium bromide.

The stability of p53 target mRNA was determined as described previously (37). Cells were treated with actinomycin D (1 μM; Sigma) for the indicated time. After treatment, total RNA was extracted, and the expression of mRNA was analyzed by RT-PCR.

**Chromatin Immunoprecipitation (ChIP) Assay**—Cells were formaldehyde cross-linked for 10 min at room temperature by adding an equal volume of cross-linking solution (1% formaldehyde in PBS) directly to the culture medium. Cross-linking was stopped by the addition of glycine to a final concentration of 125 mM. Cells were washed twice with ice-cold PBS and collected. Cells were suspended in HEPES-lysis buffer (10 mM HEPES (pH 7.5), 1% Nonidet P-40, 1 mM EDTA, 400 mM NaCl, 10% glycerol, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 3 μg/ml leupeptin, 3 μg/ml pepstatin A, and 10 μg/ml aprotinin) and were centrifuged at 11,500 rpm for 5 min at 4 °C. Nuclear pellets were resuspended in HEPES-lysis buffer, and chromatin was fragmented to an average size in the range of 600–800 bp using a Sonicator W-220 (Heat Systems Ultrasound Inc.,). Insoluble material was removed by centrifugation at 13,000 rpm for 10 min at 4 °C. The supernatant was diluted 2-fold in HEPES dilution buffer (10 mM HEPES (pH 7.5), 1 mM EDTA, 10% glycerol, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 3 μg/ml leupeptin, 3 μg/ml pepstatin A, and 10 μg/ml aprotinin) and then precleared with Protein A- and G-Sepharose beads for 2 h at 4 °C. Aliquots were stored and used for input samples. The precleared chromatin solution was incubated with blocked Protein A- and G-Sepharose beads (preincubated overnight at 4 °C with 0.7 mg/ml sonicated salmon sperm DNA and 185 mg/ml bovine serum albumin), and then either anti-p53 (1801 and DO-1) or anti-HDM2 (2A10 and SMP-14) antibodies were incubated overnight at 4 °C. The beads were washed three times in wash buffer (10 mM HEPES (pH 7.5), 0.5% Nonidet P-40, 1 mM EDTA, 200 mM NaCl, and 10% glycerol) and then resuspended in cross-link reversal buffer.
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Constitutively Expressed Tagged HDM2 Does Not Affect the Level or Constitutively Express Exogenous HDM2—To gain more insight as to how HDM2 modulates the function of p53, our goal was to generate cell clones that both contain inducible p53 and constitutively express exogenous HDM2. To distinguish between endogenous and exogenous HDM2, we used a version of HDM2 that has two FLAG epitope tags at its N terminus and an HA epitope tag at the C terminus (F-HDM2). Because tag sequences may inhibit the function of proteins, we tested the capability of F-HDM2 to interact with and degrade p53 by co-transfecting a p53 expression plasmid into H1299 cells with or without the F-HDM2 expression plasmid (supplemental Fig. 1A). As shown previously, overexpression of F-HDM2 significantly (∼50%) decreased the level of co-expressed p53 (supplemental Fig. 1A, lanes 2 and 4) (17–19). We showed previously that when higher ratios of HDM2 to p53 constructs (e.g. between 5 and 10:1) were introduced into H1299 cells there was an even greater extent of p53 degradation (38). Furthermore, when F-HDM2 was cotransfected with p53, their interaction was confirmed using an immunoprecipitation-immunoblotting assay in which p53 was immunoprecipitated by anti-FLAG antibody in the presence of F-HDM2 but not in the absence of F-HDM2 (supplemental Fig. 1B, lanes 2 and 4). Similarly, F-HDM2 was immunoprecipitated by anti-p53 antibody in the presence of p53 but not in its absence (supplemental Fig. 1B, lanes 2 and 4). Thus doubly FLAG- and HA-tagged HDM2 when overexpressed is capable of binding and degrading p53.

We used a previously well characterized H1299 clone (H24-14) expressing tetracycline-regulated wild type p53 (32). These cells express relatively low levels of p53 upon withdrawal of tetracycline that is within the range of endogenous p53 induced by DNA damage (32, 39). We established several clones expressing F-HDM2 as described under “Materials and Methods,” and one of them (H24-14/HDM2#1) that expressed relatively high levels of F-HDM2 was selected for further experiments. By using this clone, we were able to change the ratio of p53 to HDM2 protein and thereby measure the function of p53 in the same genetic background.

Constitutively Expressed Tagged HDM2 Does Not Affect the Level or Localization of p53 Protein—We first examined the levels of p53 and HDM2 in H24-14/HDM2#1 cells with and without induced p53 (Fig. 1A). As shown previously, low concentrations of tetracycline allowed (125 mM Tris-HCl (pH 6.8), 10% β-mercaptoethanol, and 4% SDS). Antibody-bound chromatin was eluted from the beads by heating at 95 °C for 30 min. The DNA was then purified using the QIAquick PCR purification kit (Qiagen). Input and immunoprecipitated DNAs were amplified by PCR. PCR was performed using the following primers: p21 (distal), 5'-CTGGGACTGGAATGTGC-3' and 5'-CTCTCAACAC-TCCCCCTCT-3'; p21 (proximal), 5'-TCTGGGTTAGCCACA-ATC-3' and 5'-CTGACATCTGACGGCTGCA-3'; PIG3 (5' upstream), 5'-AGGAGCCGAGTGTAAGGATCC-3' and 5'-AACCTCTCTGGGGCCGAGTGG-3'; PIG3 (microsatellite), 5'-GGGGCGTGCGGTT-GCCAGCCTGAG-3' and 5'-ACCTCAGGAGGACCTCAC-3'; and GAPDH, 5'-ACTATGCCTGATCTCAG-3' and 5'-CTGTGTGACTGGCGCAACTG-3'. PCR samples were resolved on 2% agarose gels.

RESULTS

Establishment of H1299 Cell Clones That Contain Inducible p53 and Constitutively Express Exogenous HDM2.

Constitutively Expressed Tagged HDM2 Does Not Affect the Level of p53 but Inhibits Expression of Select p53 Targets.—We observed that F-HDM2 and endogenous HDM2 strongly inhibited p53 degradation when the ratio of F-HDM2 to p53 was between 5 and 10:1 (Fig. 1A). As shown previously, low concentrations of tetracycline allowed (125 mM Tris-HCl (pH 6.8), 10% β-mercaptoethanol, and 4% SDS). Antibody-bound chromatin was eluted from the beads by heating at 95 °C for 30 min. The DNA was then purified using the QIAquick PCR purification kit (Qiagen). Input and immunoprecipitated DNAs were amplified by PCR. PCR was performed using the following primers: p21 (distal), 5'-CTGGGACTGGAATGTGC-3' and 5'-CTCTCAACAC-TCCCCCTCT-3'; p21 (proximal), 5'-TCTGGGTTAGCCACA-ATC-3' and 5'-CTGACATCTGACGGCTGCA-3'; PIG3 (5' upstream), 5'-AGGAGCCGAGTGTAAGGATCC-3' and 5'-AACCTCTCTGGGGCCGAGTGG-3'; PIG3 (microsatellite), 5'-GGGGCGTGCGGTT-GCCAGCCTGAG-3' and 5'-ACCTCAGGAGGACCTCAC-3'; and GAPDH, 5'-ACTATGCCTGATCTCAG-3' and 5'-CTGTGTGACTGGCGCAACTG-3'. PCR samples were resolved on 2% agarose gels.

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for a partial expression of p53, whereas more complete withdrawal of tetracycline caused a greater amount of p53 to accumulate in H24-14 (Fig. 1A) (32). As expected, endogenous HDM2 levels were increased upon induction of p53. Most interestingly, two different endogenous HDM2 polypeptides induced by p53 were resolved, although the more rapidly migrating species was predominant. In H24-14/HDM2#1 cells, F-HDM2 migrated with a very similar mobility to that of the slower mobility species of endogenous HDM2. Most surprisingly, F-HDM2 levels were increased along with the levels of induced p53 by a mechanism we do not understand. Because of this increase in F-HDM2, we estimate that at each concentration of tetracycline, ~2-fold more HDM2 (combined ectopic and endogenous) was expressed in H24-14/HDM2#1 cells compared with H24-14 cells. Although H24-14/HDM2#1 cells also expressed p53 in a tetracycline dose-dependent manner, unexpectedly, over a range of tetracycline in the medium, the levels of p53 were essentially identical to those in H24-14 cells that lacked extra HDM2. Thus the extra HDM2 apparently did not increase degradation of p53. This is not unique to this clone of H24-14 cells; when p53 levels were measured in four other stable clones, there were no differences seen at any dose of tetracycline regardless of the presence of F-HDM2 (data not shown).

Furthermore, we confirmed that stably expressed F-HDM2 was associated with p53 by performing a co-immunoprecipitation assay in which p53 was detected in the anti-FLAG antibody immunoprecipitate in the presence of F-HDM2 but not in the absence of F-HDM2 (Fig. 2A). Reciprocally, both endogenous HDM2 and F-HDM2 were immunoprecipitated by the anti-p53 antibody (Fig. 2B). Interestingly, only the faster mobility endogenous HDM2 species was immunoprecipitated by anti-p53 antibody upon withdrawal of tetracycline in H24-14 cells (Fig. 2B, lane 2). Thus, although F-HDM2 forms a complex with p53 in H24-14/HDM2#1 cells, it is unlikely to cause p53 degradation.

Because HDM2 is involved in translocating p53 from the nucleus to the cytoplasm (40, 41), we determined the intracellular localization of p53 with or without extra HDM2 by immunofluorescence (supplemental Fig. 2). Note that although it is possible to clearly discern the extra HDM2 in H24-14/HDM2#1 cells without p53 induction, the increase in detectable HDM2 when p53 is fully induced is more subtle, possibly because differences in immunofluorescence are not quantitative under these conditions. Induced p53 and endogenous HDM2 mainly accumulated in the nucleus in H24-14/HDM2#1 cells. F-HDM2 was also located in the nucleus, and induced p53 co-localized with both endogenous and FLAG-tagged HDM2 in this cell line.

Excess HDM2 Selectively Impairs Expression of Downstream Targets of p53—We examined different transcriptional targets of p53 in the presence or absence of excess HDM2. Using aliquots of the same cell extracts as in Fig. 1A, protein levels of a number of p53 targets were detected by specific antibodies (Fig. 1B). In both cell lines, the levels of these target proteins were increased along with the levels of p53 itself. Although there was no significant effect of extra HDM2 on accumulation of either p21 or Bax proteins, we observed that PIG3 and cyclin G1 (not shown) proteins were less efficiently induced in H24-14/HDM2#1 than in H24-14 cells. Note that in the experiment shown without extra HDM2, p21 and Bax were expressed at slightly greater levels than with extra HDM2 at 2.5 ng/ml tetracycline (Fig. 1B, lane 4 and 9), their levels were very similar after normalization with the amounts of β-actin. When we determined the accumulation of different p53 target mRNAs by RT-PCR analysis, the presence of extra HDM2 did not appreciably affect p21 or Bax expression, although both 14-3-3σ and PIG-3 mRNA levels were markedly reduced in cells with extra HDM2 (Fig. 1C). To clarify whether the specific reduction of PIG3 mRNA expression in the presence of excess HDM2 is a result of suppressed gene transcription or mRNA stability, we examined the rate of p21 and PIG3 mRNA decay with or without excess HDM2 following inhibition of nascent RNA transcription by actinomycin D. In both cell lines, the decay of p21 mRNA was very similar at distinct time points over a 12-h period (Fig. 1D). Although PIG3 mRNA was less well induced in the presence of extra HDM2, the decay of its mRNA was very similar with and without excess HDM2 (Fig. 1D). These data indicate that extra HDM2 suppressed PIG3 induction via transcriptional repression. Furthermore, of three other H24-14-derived clones expressing significant levels of F-HDM2, none showed differences in p21 induction, whereas PIG3 levels were reduced in each case (data not shown). Thus excess HDM2 exerts a promoter-selective effect on p53 activity at the transcriptional level.

Excess HDM2 Affects Cell Growth and Suppresses Cell Death Induced by p53 and DNA-damaging Agents—Although we could not see any differences in the levels and localization of p53 between H24-14 and H24-14/HDM2#1 cells, we observed that the latter showed alterations in morphology with respect to the parental H24-14 cells after induction of p53 being somewhat more rounded (data not shown). To characterize the participation of HDM2 in the cellular functions of p53, we compared cell cycle distribution between these two cell lines. As demonstrated previously, the maximal induction of p53 in H24-14 cells resulted in increased populations of cells in G1 and in G0/M with decreased numbers of S phase cells (from ~36 to 5%) indicating that the expression of p53 in H24-14 cells caused a predominant G1 arrest with some G2 arrest (from 14 to 22%) (Fig. 3A) (32, 39). By contrast, the induction of p53 in H24-14/HDM2#1 cells led to a significant increase in G0/M cells (from 15 to 37%). Although the percent of cells in S phase was also decreased by the expression of p53 in the H24-14/HDM2#1 clone, the extent of the reduction was slightly lower than in the H24-14 parental cells. Of four other F-HDM2-expressing H1299-14 clones tested, each showed relatively increased G2 arrest with the most pronounced being in the H24-14/HDM2#1 clone (37%). Specifically, com-
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**A**

| p53 - | p53 + | Sub-G1 | G1 | S | G2/M |
|-------|-------|--------|----|---|------|
| H24-14 | p53 - | 1.2 ± 0.2 | 49.2 ± 4.9 | 35.8 ± 0.8 | 14.0 ± 5.2 |
| H24-14/HDM2#1 | p53 - | 3.5 ± 2.6 | 74.5 ± 3.2 | 34.5 ± 2.6 | 14.5 ± 3.7 |
| H24-14 | p53 + | 8.8 ± 2.0 | 64.6 ± 5.2 | 4.9 ± 2.5 | 21.7 ± 1.3 |
| H24-14/HDM2#1 | p53 + | 4.8 ± 1.4 | 49.8 ± 2.4 | 8.3 ± 1.0 | 37.1 ± 3.2 |

**B**

**FIGURE 3.** Excess HDM2 increases G2 arrest and suppresses cell death induced by DNA damage. **A,** 48 h after removal of tetracycline, cells were fixed and stained with propidium iodide and processed for FACS analysis. Percentages of cells in the G1, S, and G2/M phases of cell cycle under each condition were calculated and tabulated. Data represent results from three independent experiments (mean ± S.D.). **B,** 48 h after removal of tetracycline, the cells were treated with daunorubicin (DAUNO; 100 ng/ml). Thirty-six h later, the cells were fixed and stained with propidium iodide and processed for FACS analysis. Numbers shown are % cells with sub-G1 DNA content.

pared with 22% of cells in clone H24-14 in G2/M, different clones expressing F-HDM2 had 25, 28, 27, and 31% of their cells in G2/M when p53 was induced (data not shown). These data suggest that extra HDM2 partially suppresses p53-mediated G2 arrest but the cells arrest in G2.

We next determined the extent of apoptosis with or without extra HDM2. We reported previously that apoptosis in H24-14 cells is minimal and is only modestly increased when either p53 is induced or cells are treated with a number of chemotherapeutic agents in the absence of p53 (32, 39). By contrast, there is a synergistic increase in the extent of cell death when these cells both express p53 and are subjected to these agents. We used this approach to determine whether the presence of extra HDM2 can affect DNA damage-facilitated apoptosis mediated by p53 (Fig. 3B). To induce apoptosis, 48 h after removal of tetracycline, the cells were treated with the DNA-damaging agent, daunorubicin (100 ng/ml), and 36 h later the cells were analyzed by flow cytometry. As expected, induction of p53 in the absence of DNA damage led to only a moderate increase in the amount of sub-G1 cells in both cell lines (Fig. 3B). Treatment with daunorubicin in the absence of p53 also caused a modest sub-G1 increase in both cells compared with untreated cells. However, as we had observed previously, the combination of the induction of p53 with DNA damage significantly increased the number of H24-14 cells with sub-G1 DNA content. Notably, DNA damage led to markedly less p53-mediated apoptosis in H24-14/HDM2#1 cells. Moreover, constitutively expressed F-HDM2 also suppressed p53-mediated apoptosis induced by another DNA-damaging agent, neocarzinostatin (data not shown). These data suggest that extra HDM2 inhibits cellular functions of p53 not through destabilization or changing the localization of p53 but most likely through its ability to selectively repress p53 transcription functions.

**Decreased p53 Acetylation in Cells Expressing Extra HDM2 Is Not Correlated with Altered p53 or HDM2 Association with p53 Target Promoters**—It is likely that the activity of p53 is regulated through post-translational modifications (3, 6, 29), and HDM2 is a potential inhibitor of the acetylation of p53 (25, 27, 28). We therefore tested whether either phosphorylation or acetylation of p53 at specific sites was altered in the presence of extra HDM2. We used unstressed cells because with the exception of the experiment on apoptosis shown in Fig. 3B, our experiments were carried out in the absence of DNA-damaging treatments. Furthermore, we have detected previously the modification of p53 using phospho-specific antibodies in untreated H24-14 cells that is increased upon treatment of cells with genotoxic agents (Ref. 39 and data not shown). Indeed, although the extent of phosphorylation of p53 at its N terminus at Ser-15 and its C terminus at Ser-392 was very similar between both cell lines, we observed that acetylation at Lys-382 was significantly decreased in H24-14/HDM2#1 cells (Fig. 4).

Because acetylation of p53 may increase the DNA binding affinity of p53 for DNA under some conditions (42, 43), we considered the possibility that extra HDM2 might differentially affect p53 binding to individual promoters. To test this possibility, we performed ChIP assays. After induction of p53, cells were cross-linked, and p53-chromatin complexes were immunoprecipitated with either anti-p53 and anti-HDM2 antibodies or anti-phospho-Ser-15, anti-phospho-Ser-392, or anti-acetyl-Lys-382 antibodies as indicated.
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**FIGURE 5.** Excess HDM2 does not affect DNA binding of p53 in vivo. Cells were grown in the presence of indicated amounts or the absence of tetracycline (Tet) for 48 h and treated with formaldehyde. Chromatin extracts were prepared as outlined under "Materials and Methods." Immunocomplexes from the extracts were isolated with either anti-p53 antibody (PAb1801 and DO-1) or anti-HDM2 antibody (SMP-14 and 2A10). The DNA isolated from each immunocomplex was amplified by PCR using primers specific for the p53-response elements located 1.4 kb (p21 (proximal)) and 2.3 kb (p21 (distal)) upstream of the transcription initiation site in the p21 promoter, and for the p53-response elements located 1.4 kb (PIG3 (proximal)) and 0.5 kb downstream (PIG3 (micro.)) of the transcription initiation site in the PIG3 promoter. Total DNA isolated from chromatin extracts under each condition was similarly amplified by PCR and used as input controls.

from the immunoprecipitates, p53 binding regions within the p21 and PIG3 promoters were amplified by PCR. The p21 promoter contains proximal and distal p53-response elements, located 1.4 and 2.3 kb upstream of the transcription start site, respectively (44, 45). We observed that binding to the two p53 sites at the p21 promoter by p53 closely matched the pattern of mRNA expression in both cell lines (Fig. 5A). Two p53-response elements have also been identified in separate regions of the PIG3 gene located between −328 and −309 (46) and another microsatellite region between +442 and +516 (47). Although PIG3 mRNA was consistently less well induced in the presence of extra HDM2, ChIP analysis showed that p53 binding to the two p53 sites at the PIG3 promoter was very similar with and without excess HDM2 (Fig. 5B). Even at lower levels of p53 (2.5 ng/ml of tetracycline), similar results were obtained. Thus HDM2 must differentially affect individual p53-responsive promoters through other mechanism(s). We therefore considered the possibility that HDM2 might be relatively more efficiently associated with p53 at those target promoters that were affected by its overexpression. To test this we examined the presence of HDM2 by ChIP at the same p21 and PIG3 p53 genomic binding sites. We found that there was not relatively more HDM2 associated with the PIG3 sites than with the p21 sites. In the same experiment when we examined the accumulation of PIG3 and p21 mRNA by RT-PCR, there was again clearly markedly less PIG3 mRNA expressed in the H24-14/HDM2#1 cells than in the H24-14 cells, although there was not a significant difference in the extent of p21 mRNA detected in either cell line (data not shown). Semi-quantitative RT-PCR does not provide sufficiently accurate data to evaluate possible real but subtle differences in the amount of protein bound to a given site. Nevertheless, when we used densitometry to analyze the data comparing p53 or HDM2 association with either p21 or PIG3 promoters with or without extra HDM2, any differences recorded were not statistically significant (data not shown). Our data indicate that HDM2 neither affects the binding of p53 to its target sites in promoters nor associates selectively with p53 at such target sites. Thus, the presence of HDM2 at such sites is not sufficient to account for the difference in expression of p53 target genes seen when extra HDM2 is present in cells.

**DISCUSSION**

In this study, we utilized a cell line expressing inducible p53 and constitutive HDM2 to characterize how HDM2 regulates the function of p53 under physiological conditions. Most previous reports describing the functional regulation of p53 by HDM2 have relied on either transient overexpression or in vitro assays. Our cell line expresses relatively low levels of p53 that are well within the range of endogenous p53 levels that accumulate when cells are subjected to stresses such as DNA damage (31). Moreover, in these cells, there is only a 2-fold increase in the total amount of HDM2 compared with the amounts of endogenous HDM2 that is induced upon expression of p53 in the parental H24-14 cells. This is in line with studies in which levels of endogenous HDM2 are increased because of SNP309 in the human p53 promoter (31). Our study confirms and extends their findings with SNP309 in an isogenic setting. Using this system, we observed that degradation of p53 is not the dominant mode by which HDM2 inhibits the function of p53 under physiological levels in H1299 cells. In contrast, HDM2 inhibits the acetylation of p53, and under-acetylated p53 displays altered cell cycle regulation, reduced apoptosis, and altered promoter selectivity without changing binding of p53 to the promoter regions that were examined. Our data were confirmed in other derived clones of H24-14 cells expressing F-HDM2. Thus, the primary role of HDM2 appears to be inhibition of the transcription function of p53 in these cells.

We were somewhat surprised that the extra HDM2 did not appear to cause detectably increased degradation of p53. One explanation why excess HDM2 did not change the stability of p53 is because of the difference of the ratio of p53 to HDM2 proteins. Indeed, we confirmed the results of others that transiently overexpressed HDM2 enhanced destabilization of p53 (17, 18). Because transient transfection can lead to a much higher per cell level of proteins, and in our cells there is only a 2-fold relative increase in total HDM2 protein, this is the most likely explanation. It also is possible that the artificial promoter for p53 in these cells is relatively stronger than the endogenous p53 promoter and the levels of p53 protein being synthesized would be correspondingly greater. Thus, the production of p53 protein might be dominant over the degradation of p53 in these cells even with double the amount of HDM2. We also considered that these cells might have deficiencies in proteasome-mediated protein degradation. Arguing against this, when they were treated with the proteasome inhibitor MG-132, the levels of exogenous p53 and HDM2 were increased (data not shown). Furthermore, it was recently reported that human cell lines with naturally
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occurring excess HDM2 do not appear to have relatively less p53 (48). Importantly, even with the modestly increased level of HDM2 in our system p53 activity can be profoundly affected.

Our experiments showed that excess HDM2 alters cell cycle arrest and prevents apoptosis induced by p53. Several downstream targets of p53 involved in cell cycle arrest have been identified, including p21 (49), gadd45 (50), 14–3–3-ζ (51), and reprimo (52). Additionally, at least 15 different p53 target genes have been shown to promote apoptosis (3). Of those involved in cell cycle regulation, we found that the levels of p21 did not change with or without excess HDM2 but that 14–3–3-ζ was reduced in the presence of excess HDM2. Recently, it was shown that another p53 target, cyclin G1 (53) can potentially induce G1 arrest when overexpressed (54). Because the levels of cyclin G1 protein were also significantly reduced with extra HDM2 (data not shown), one possibility is that G1 arrest is weakened with excess HDM2, and consequently G2 arrest might be increased. Most interestingly, however, there was little or no difference in cyclin G1 mRNA levels with or without extra HDM2 upon induction of p53, suggesting that extra HDM2 can affect processes other than transcription (data not shown). Regarding p53 regulation of pro-apoptotic genes, although there was no significant effect on Bax activation activity of p53, suggesting that extra HDM2 can affect processes other than transcription (data not shown). Regarding p53 regulation of pro-apoptotic genes, although there was no significant effect on Bax activation activity of p53, suggesting that extra HDM2 can affect processes other than transcription (data not shown). Regarding p53 regulation of pro-apoptotic genes, although there was no significant effect on Bax

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