Regulation of p53 Stability and Function in HCT116 Colon Cancer Cells*

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Matthias D. Kaeser‡, Stephanie Pebernard‡, and Richard D. Iggo§

From the Oncogene Group, Swiss Institute for Experimental Cancer Research, Ch des Boveresses 155, 1066 Epalinges, Switzerland

We have used a lentiviral vector to stably express p53 at a physiological level in p53 knockout HCT116 cells. Cells transduced with wild type p53 responded to genotoxic stress by stabilizing p53 and expressing p53 target genes. The reconstituted cells underwent G1 arrest or apoptosis appropriately depending on the type of stress, albeit less efficiently than parental wild type cells. Compared with cells expressing exogenous wild type p53, the apoptotic response to 5-fluorouracil (5FU) was >50% reduced in cells expressing S15A or S20A mutant p53, and even more reduced by combined mutation of serines 6, 9, 15, 20, 33, and 37 (N6A). Among a panel of p53 target genes tested by quantitative PCR, the gene showing the largest defect in induction by 5FU was BBC3 (PUMA), which was induced 4-fold with wild type p53 and 2-fold by the N6A mutant. Mutation of N-terminal phosphorylation sites did not prevent p53 stabilization by doxorubicin or 5FU. MDM2 silencing by RNA interference activated p53 target gene expression in normal fibroblasts but not in HCT116 cells, and exogenous p53 could be stabilized in HCT116 knockout cells despite combined mutation of p53 phosphorylation sites and silencing of MDM2 expression. The MDM2 feedback loop is thus defective, and other mechanisms must exist to regulate p53 stability and function in this widely used tumor cell line.

The p53 tumor suppressor gene is mutated in over 50% of human tumors and plays an important role in the response to genotoxic stress and hypoxia (1). p53 responds to upstream signals by activating transcription of genes important for cell cycle arrest, DNA repair, and apoptosis. The spectrum of target genes induced depends on the type of stress and the tissue analyzed (2). In addition to its transcriptional activity p53 also induces apoptosis by non-transcriptional mechanisms, including inhibition of Bcl-2 and Bcl-XL at the mitochondrial membrane (3). Upstream regulators of p53 function include the MDM2 oncogene and the p14ARF tumor suppressor gene (4, 5). MDM2 is a p53 target gene that acts as a negative feedback loop to inhibit p53 by blocking the access of transcription cofactors to the p53 transactivation domain (6) and by inducing p53 degradation (7, 8). MDM2 is a ring finger E3 ligase that mono-ubiquititates p53. This leads to p53 poly-ubiquitination by p300, and it is the poly-ubiquitinated form that is degraded by the proteasome (5, 9, 10). The second major type of regulation of p53 is phosphorylation by kinases, including ATM, ATR, chk1, chk2, JNK1, CDK1, CDK2, casine kinases 1 and 2, protein kinase C, and the COP9 signalosome-associated kinase (reviewed by Anderson and Appella (11)). Kinases targeting the N-terminal transactivation domain are of particular interest, because they can regulate the recruitment of transcription cofactors and the binding of MDM2. The most intensively studied sites are serines 15, 20, 33, and 37. In response to DNA damage, Ser-15 is phosphorylated by the ATM and ATR kinases, and Ser-20 by the chk1 and chk2 kinases. Ser-15 phosphorylation is thought to regulate binding of transcription cofactors like the p300/CBP histone acetyltransferase (12) and Ser-20 to regulate the binding of MDM2 (13). Phosphorylation of Ser-33 and Ser-37 leads to acetylation of Lys-382 by p300 (14). Several other histone acetyltransferase-containing complexes have been implicated in transcriptional activation by p53, including p300/CBP-associated factor, transformation/transcription domain-associated protein, and general control of amino acid synthesis 5-like protein complexes (14–16).

The model that emerges from these studies is that phosphorylation plays a crucial role in regulating the stability and activity of p53. Against this must be set a large body of evidence from mutagenesis studies showing little or no effect of mutating p53 phosphorylation sites (17, 18). There is no doubt that the sites are phosphorylated in vivo in response to stress, as has been demonstrated repeatedly by 32P-labeling and by Western blotting with phospho-specific antibodies (11, 19). One possible explanation for the discrepancy is that the assays used to test p53 function employed overexpression systems or cells with constitutive or defective signaling to p53. Tumor cells lacking p53 function frequently activate transfected wild type p53 constitutively, either as a result of ongoing DNA damage or as a side effect of the transfection procedure. To avoid this problem, one can use cells that have retained wild type p53. It is possible to see regulation of the stability of exogenously supplied p53 mutants in these cells, but it is difficult to test the transcriptional activity or apoptotic function of the mutants because of the presence of the resident wild type allele (18). Finally, it is now possible using knock-in mouse technology to test the activity of specific mutants expressed from the endogenous locus. This approach has only been applied to a small number of phosphorylation sites, but 15A and 20A knock-in mice (equivalent to 15A and 20A in human p53) are not spontaneously tumor prone, suggesting that phosphorylation on these sites is not essential for the tumor suppressor activity of p53 (20, 21).

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‡ Both authors contributed equally to this work.
§ To whom correspondence should be addressed. Tel.: 41-21-692-5889; Fax: 41-21-652-6933; E-mail: Richard.Iggo@isrec.unil.ch.

The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; JNK1, c-Jun NH2-terminal kinase 1; CDK1,2, cyclin-dependent kinase 1 and 2; HLF, Human diploid lung fibroblast; RT, reverse transcriptase; RNAi, RNA interference; RSV, Rous sarcoma virus; 5FU, 5-fluorouracil; PI, propidium iodide; FdUMP, 5-fluoro-2'-deoxyuridine 5'-monophosphate; ATM, ataxia telangiectasia mutated; chk1,2, checkpoint kinase 1,2; ATR, ATM-related; shRNA, short hairpin RNA.
To address these issues, we have used a lentiviral vector to stably express p53 regulatory site mutants at a normal, physiological level. We used a cell line that was derived from wild type p53-containing HCT116 cells by gene disruption. By definition, the parental cells tolerate the presence of wild type p53, and several studies have shown that they respond appropriately to diverse stresses (22, 23). We show that exogenous p53 in these cells can be stabilized, activate p53 target genes, and induce cell cycle arrest and apoptosis in response to stress, and we describe the phenotype of classic p53 regulatory mutants in these cells.

MATERIALS AND METHODS

Cell Culture and Antibodies—HCT116 and p53-null derivatives were supplied by Dr. B. Vogelstein (23). Human diploid lung fibroblasts (HLFs) were supplied by Dr. M. Nahholz. D07 mouse hybridoma cells were supplied by Dr. D. Lane (24). The primary antibodies used for immunoblotting were D07 and PL399 (Santa Cruz Biotechnology, Santa Cruz, CA) for p53, C-19, for p21 (Santa Cruz Biotechnology) and C-25 or 46 (Santa Cruz, CA) for p53, C-19, for p21 (Santa Cruz Biotechnology) and PAb240 (Upstate Biotechnology, Lake Placid, NY) for p53. The secondary antibodies used for immunoblotting were goat anti-rabbit IgG, donkey anti-goat IgG, and donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Primary antibodies used for immunofluorescence were DO7 and FL393 (Santa Cruz Biotechnology, Santa Cruz, CA) for p53, C-19, for p21 (Santa Cruz Biotechnology) and C-25 or 46 (Santa Cruz, CA) for p53, C-19, for p21 (Santa Cruz Biotechnology). The primary antibodies used for immunohistochemistry were DO7 and FL393 (Santa Cruz Biotechnology, Santa Cruz, CA) for p53, C-19, for p21 (Santa Cruz Biotechnology) and C-25 or 46 (Santa Cruz, CA) for p53, C-19, for p21 (Santa Cruz Biotechnology). Human diploid lung fibroblasts (HLFs) and HCT116 cells were infected at a multiplicity of 5–10 infectious units per cell.

RESULTS

Exogenous p53 Responds to DNA Damage in p53 Knockout HCT116 Cells—Wild type p53 was cloned into a lentiviral vector under the control of the RSV promoter (Fig. 1A). p53 knockout derivatives of HCT116 colon tumor cells were infected with this virus, and transduced cells were selected with puromycin. For simplicity, “parental wt” in the figures refers to endogenous wild type p53 in parental HCT116 cells; all other forms are expressed from integrated lentiviruses in p53 knockout cells. The RSV promoter construct was chosen because integration of a single copy of the virus in these cells gave a level of expression of exogenous p53 similar to that of parental wt p53 (Fig. 1B). The difference in migration of the two p53 forms is explained by the codon 72/R polymorphism (see below). The level of MD2 and p21 was substantially higher in p53-transduced HCT116 cells than in parental wt cells. The level of p21 was also substantially higher in p53-transduced and parental wt cells. Finally, the biological consequences of p53 expression in the p53-null cells was tested by flow cytometry. In untreated cells, the cell cycle distribution was similar in the presence and absence of p53 (Fig. 1E, control). In parental wt cells, the topoisomerase II inhibitor doxorubicin induced G1 and G2 cell cycle arrest, whereas 5FU induced apoptosis (Fig. 1E, parental wt). Knockout cells transduced with control virus failed to undergo G1 arrest or apoptosis in response to these treatments (Fig. 1E, null). Transduction of wild type p53 restored both the G1 arrest response to doxorubicin and the apoptotic response to 5FU (Fig. 1E, wt), although the magnitude of the response was reduced compared with the parental wild type cells. A trivial explanation for the difference is that the exogenous p53 has been expressed to a lower level.

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proline at codon 72, whereas the parental cells have arginine (31). To rule this out, both p53 isoforms were tested in the lentiviral system. The stability, inducibility, and apoptotic phenotype of the two forms were similar in all of the assays performed (Fig. 2).

In summary, p53-null HCT116 cells transduced with a lentiviral vector expressing wild type p53 respond to DNA-damaging and chemotherapeutic agents by stabilizing p53 and inducing expression of p53 target genes. They undergo G1 arrest or apoptosis appropriately depending on the type of stress, but the response is weaker than in parental wt cells.

Response of Phosphorylation and Acetylation Site Mutants to Doxorubicin—Because exogenous p53 can respond to DNA damage signals in the p53 knockout cells, the signaling pathways upstream of p53 must be largely intact in these cells. Indeed, Saito et al. (19) recently tested p53 for modification at 12 different sites in parental wt HCT116 cells using phosphorylation and acetylation-specific antibodies and showed that all of the sites mutant in our constructs are modified in response to genotoxic stress. To test the role of different modifications in HCT116 cells, the following mutants were cloned into the lentiviral vector: single mutants S15A, S20A, and K382R; a double mutant S33A/S37A; and a sextuple mutant S6A/S9A/S15A/S20A/S33A/S37A (“N6A”). None of the mutations prevents MDM2 binding (18). The tumor mutant 175H was used as a negative control: it fails to transactivate p53 target genes and is usually stable in the absence of DNA damage signaling in cells lacking p53 function.

Doxorubicin was used to introduce double strand DNA breaks. These activate ATM and chk2, which directly phosphorylate Ser-15 and Ser-20 (11). Two antibodies were used to test the p53 level: a polyclonal antibody that recognizes all forms of p53, and the monoclonal antibody DO7 whose binding is known not to be affected by post-translational modifications (13). The level of all of the p53 mutants except 175H increased after doxorubicin treatment (Fig. 3A; the 20A mutation destroys the DO7 epitope, explaining the absence of signal with this mutant and N6A). The mutant showing the best inducibility was 20A (2.9-fold increase in p53 level after doxorubicin treatment, versus 3.5-fold for parental wt, 1.9-fold for exogenous wt, and 1.5- to 2.3-fold for the other mutants). The good response of 20A is paradoxical, given the postulated role of serine 20 phosphorylation in blocking MDM2 binding. All of the regulatory site mutants were able to induce p21 and MDM2 expression, although none was as efficient as parental wild type p53 (Fig. 3A). In particular, the good inducibility of the 20A mutant at the p53 protein level did not result in a better induction of p21 or MDM2. Cells containing the regulatory site mutants showing the least inducibility of p21 and MDM2 still expressed more p21 and MDM2 in the uninduced state than the 175H mutant, indicating that even low levels of these mutants have demonstrable transcriptional activity. Flow cytometry was used to test the biological activity of the mutants. As described above, parental wt cells gave 2-fold more G1 arrest than the cells transduced with wild type p53. The correct control for assessment of the contribution of the regulatory sites is thus the cells transduced with the lentiviral wild type vector, not the parental wt cells. Despite the lower induced p21 level with some mutants (range 0.5 to 0.9 relative to the p21 level with exogenous wt), all except 175H were able to induce G1 arrest to a
similar level as was seen with exogenous wild type p53 (Fig. 3B).

In summary, mutation to alanine of Ser-15 and Ser-20, which are highly conserved and known to be phosphorylated by ATM and chk2 in response to DNA double strand breaks, does not prevent p53 stabilization following doxorubicin treatment. Mutation of either residue, as well as mutation of K382 to a non-acetylatable form, causes a small reduction in the transcriptional response detected by Western blotting for p21 and MDM2. None of the mutations tested can abolish the G1 arrest response, although it is important to note that the response in all cases was weaker than in the parental wt cells. These results are compatible with phosphorylation of Ser-15 and Ser-20 and acetylation of Lys-382 playing a role in the transcriptional response detected by Western blotting for p53 after translation inhibition. Cells were harvested at the indicated times after cycloheximide addition. D, cell cycle distribution measured by PI staining. Cells were treated with 100 ng/ml doxorubicin for 24 h or 0.38 mM 5FU for 30 or 36 h.

Response of Phosphorylation and Acetylation Site Mutants to 5FU—Cells expressing the same mutants were then tested with 5FU. All except the 175H control were stabilized following drug treatment (Fig. 4A). p21 and MDM2 were induced to a similar extent as in the parental wt cells (Fig. 4A). Flow cytometry showed defects in apoptosis induction by the 15A, 20A, N6A, and 382R mutants (Fig. 4B). Relative to exogenous wild type p53, individual mutations produced a >50% reduction in the apoptotic response, whereas the combined N6A mutations reduced apoptosis to the background level seen in the p53-null cells. Because the apoptotic response of the 33A/37A mutant was the same as with exogenous wild type p53, the loss of activity of the N6A mutant is most likely due to the combined effect of the 15A and 20A mutants. A biological role for acetylation of lysine 382 is suggested by the partial loss of apoptosis induction by this mutant. Because the effects were only partial, the experiments were repeated using cells infected at a higher multiplicity of infection, resulting in a higher starting level of p53. The level of apoptosis increased with the p53 level, confirming that p53 function is only partially impaired by the mutations (data not shown).

p21 and MDM2 do not induce apoptosis, so the normal induction of these genes by the N6A mutant does not rule out a transcriptional basis for the apoptotic defect of this mutant. To identify possible transcriptional mediators of the effect, we selected a group of genes induced on microarrays by wt p53 in these cells plus some likely candidates culled from the literature, and examined their induction by the N6A mutant using quantitative PCR. The basal level of expression of all of the genes tested was slightly lower with the N6A mutant (Table I, Basal). This could be explained by a difference in expression of the wt and N6A p53 from the integrated lentiviruses, but the ratio of the p53 mRNA levels measured in the same samples was 1.0, suggesting that the transduced cell populations were well matched. There were small differences in the inducibility of the genes tested, although all could still be induced (Table I). The

**Fig. 2.** Comparison of 72R/P isoforms. A, p53, MDM2, and p21 induction after doxorubicin treatment. Cells expressing p53 72P or 72R were treated with 200 ng/ml doxorubicin for the indicated times. B, p53, MDM2, and p21 induction after 5FU treatment. Cells expressing p53 72P or 72R were treated with 0.38 mM 5FU for the indicated times. C, measurement of p53 stability by Western blotting for p53 after translation inhibition. Cells were harvested at the indicated times after cycloheximide addition. D, cell cycle distribution measured by PI staining. Cells were treated with 100 ng/ml doxorubicin for 24 h or 0.38 mM 5FU for 30 or 36 h.

**Fig. 3.** Target gene expression and G1 arrest in response to doxorubicin. A, Western blot for p53, p21, and MDM2. Cells were untreated (−) or treated (+) with 200 ng/ml doxorubicin for 9 h. The S20A mutation disrupts the epitope of DO7. B, cell cycle distribution measured by PI staining. Cells were mock treated or exposed to 100 ng/ml doxorubicin for 24 h.

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* M. D. Kaeser, S. Pebernard, and R. D. Iggo, unpublished data.
The largest difference was in the induced level of the **BBC3** gene (**PUMA**), which has previously been implicated in apoptosis induction by p53 in HCT116 cells (32–34).

In summary, relative to exogenous wild type p53, mutation of N-terminal p53 phosphorylation sites leads to selective defects in apoptosis induction by 5FU but does not abolish p53 stabilization or transactivation of most p53 target genes. The gene most affected by the mutations was **PUMA**, a gene suspected to play an important role in apoptosis induction in these cells (35), although even **PUMA** induction was reduced but not abolished.

**Role of MDM2 in Stabilizing p53**—The above results show that p53 can be stabilized by doxorubicin and 5FU despite mutation of serine 20 to a residue that can not be phosphorylated. This is incompatible with the model that serine 20 phosphorylation by chk2 protects p53 from MDM2 binding (13, 36). Alternatively, there may be redundant signaling by DNA damage checkpoint proteins to both p53 and MDM2 (37, 38). To distinguish between these models, MDM2 expression was blocked by RNA interference. Short hairpin (sh) oligonucleotides targeting MDM2 were cloned into a lentiviral vector and expressed from a polymerase III promoter. Three different shRNAs were used to target MDM2: two target the 3' untranslated region, the third targets a sequence in the p53 binding domain near the N terminus. MDM2 is expressed from a constitutively active promoter (P1) and a p53-inducible promoter (P2) located in the first intron of the P1 transcript. All three shRNAs target regions that are common to both transcripts. None of the vectors induced an interferon response (data not shown) (28). The response to MDM2 silencing was tested in HCT116 cells and in normal human embryonic lung fibroblasts (HLFs). Western blotting showed that all three shRNAs substantially reduced MDM2 expression in both cell lines (Fig. 5A).

Destruction of MDM2 transcripts by RNAi should lead to transactivation of p53 target genes by interruption of the MDM2 feedback loop. This should result in an increase in the fraction of MDM2 transcripts initiated at the P2 promoter. In HLFs the level of P1 transcripts fell while that of the P2 transcripts rose after targeting with MDM2 shRNA, demonstrating that the feedback loop is active in these cells (Fig. 5B). This was accompanied by an increase in p21 level measured by RT-PCR (Fig. 5B). The p53 level did not change, suggesting that occlusion of the transactivation domain rather than p53 degradation is the major mechanism of MDM2 inhibition of p53 in these normal cells (Fig. 5A). In contrast, the level of both the P1 and P2 transcripts declined in HCT116 cells infected with the MDM2 shRNA viruses (Fig. 5B). The p53 level did not change (Fig. 5A), and p21 transcription was not activated (Fig. 5B). This suggests that p53 may not be under stringent MDM2 control in HCT116 cells.

To determine whether the stabilization of p53 by DNA-damaging agents in HCT116 cells is also independent of MDM2, p53 knockout cells expressing MDM2 shRNA A or C were transduced with lentiviruses expressing wild type p53 and the 20A, N6A, or 22Q/23S p53 mutants. The 22Q/23S mutant is unable to activate transcription or bind to MDM2 and behaves...
like a p53 null when knocked into the p53 locus in mice (39, 40). Western blotting showed that the level of the 22Q23S mutant was unresponsive to DNA damage, as expected (Fig. 6). In the cells infected with shRNA control vector, MDM2 was detectable by Western blotting prior to drug treatment, and the level rose substantially after treatment (Fig. 6). In the MDM2 shRNA-expressing cells, MDM2 protein was undetectable before stress and rose to a barely detectable level after stress. The MDM2 shRNAs were thus able to degrade both basal and induced MDM2 transcripts. The reduction in MDM2 protein level did not alter the response of exogenous wild type p53 or the phosphorylation site mutants to either 5FU or doxorubicin (Fig. 6). Neither N-terminal p53 phosphorylation nor MDM2 binding are thus required for p53 stabilization in response to doxorubicin or 5FU in these cells.

Role of Pirh2 in Stabilizing p53—Leng et al. (41) recently reported that ZNF363 (Pirh2) is a p53 target gene that induces p53 degradation. Because we could not see an effect of MDM2 on p53 stability in HCT116 cells, we tested whether Pirh2 might be regulating p53 stability in these cells. Hairpin oligonucleotides targeting Pirh2 were cloned into a lentiviral vector as for MDM2. The efficiency of targeting was assessed by quantitative PCR for Pirh2 mRNA. Out of five vectors tested, one could reduce Pirh2 to ~10% of its starting level (Fig. 7A). The Pirh2 level was not checked at the protein level, but the shRNA targets the region of the mRNA encoding the p53 binding domain. Hence, any alternately spliced forms that escaped cleavage by the small interfering RNA should be unable to induce p53 degradation. Silencing of Pirh2 expression was not accompanied by an increase in the level of p21 or P2-specific MDM2 transcripts (Fig. 7A), and there was no change in the p53 level (Fig. 7B), indicating that in the conditions tested Pirh2 does not regulate p53 stability in these cells.

**DISCUSSION**

Many previous studies have cast doubt on the importance of p53 phosphorylation for regulation of p53 level or function. In this study, we have shown that neither p53 phosphorylation on N-terminal serines nor MDM2 expression is required for stabilization of exogenous p53 in response to genotoxic stress in HCT116 cells. This widely used cell line is mismatch repair-deficient and has a near diploid genome. These properties make it an attractive substrate for knockout of human genes, such as p53, p21, bax, FDXR, chk2, and PUMA (23, 35, 42–44). p53 is frequently not regulated correctly in cells that have spontaneously mutated it, which makes the study of p53 regulation difficult or impossible. By using cells in which the p53 gene was deliberately knocked out, we hoped to avoid this type of problem. An alternative would have been to use tumor cells that retain wild type p53, but it is difficult in these cells to separate the phenotype of experimentally introduced p53 mutants from that of the resident p53 gene. HCT116 cells have an increased point mutation rate, which probably explains why some aspects of p53 function were abnormal after reintroduction of exogenous wild type p53. In particular, this may explain the reduced G1 arrest and apoptotic activity of exogenous wild type p53 in the knockout cells relative to parental wt cells.

Lentiviral delivery results in stable p53 expression for at least 11 passages, which is far more than we have previously seen with oncoretroviral vectors. We compared lentiviral vectors expressing transgenes from the CMV, EF1α, RSV, and PGK promoters to identify the appropriate construct for expression of p53 at the same level as in the parental wt HCT116 cells. Because lentivirally transduced cells express transgenes more uniformly than stably transfected cells, there is no need to clone the cells to obtain consistent results. During the course of this study, it was reported that the 72P allele of p53 induces apoptosis less efficiently than the 72R allele (31), but we could not see a clear difference between the two alleles in our system.

Several groups have previously reported that Ser-20 phosphorylation by chk2 blocks MDM2 binding and thereby stabilizes p53 in response to DNA damage (13, 36, 45). Other groups have reported a lack of effect of Ser-20 mutation or chk2 deletion on the p53 response in human cells (18, 44), and knock-in mice with alanine at the mouse equivalent of Ser-20 have no defects in p53 function (21). In keeping with the latter view, we can see no defect in stabilization of the 20A mutant. The p53 level in unstimulated cells was lower with this mutant, consistent with a previous report showing that 20A p53 is more sensitive to degradation by MDM2 (46). Despite its lower level in unstimulated HCT116 cells, the 20A mutant was stabilized normally after DNA damage. By definition, phosphorylation of Ser-20 can not mediate the stabilization of the 20A protein, because alanine cannot be phosphorylated. A plausible interpretation would be that the checkpoint pathway signals redundantly to both p53 and MDM2. This possibility is supported by studies showing changes in phosphorylation of MDM2 following DNA damage (37, 38). To test this model, we inhibited MDM2 expression using RNAi. This showed that p53 stabilization could still occur after silencing of MDM2. With the best shRNA, the level of the P1 transcript was reduced to 16% and the P2 transcript to 30% of the starting level in parental wt cells, and the MDM2 protein level was substantially reduced. This reduction did not lead to activation of p21 transcription. The lack of response to MDM2 RNAi in HCT116 cells contrasts with that in normal fibroblasts, where p53-dependent transcription was activated by the full in MDM2 level. Antibodies and peptides that block the binding of p53 to MDM2 have
previously been shown to stabilize p53 and activate transcription of p53 target genes in normal cells (47–50). p53 activation by RNAi in fibroblasts led to an increase in transcription of p21 and MDM2 P2 transcripts, demonstrating that the RNAi vector could have the expected effect in cells with a functioning MDM2 feedback loop. There was no change in p53 level in the fibroblasts, which seems to indicate that in the conditions tested, MDM2 exerts its effect through occlusion of the transcription activation domain rather than by inducing p53 degradation. We conclude that p53 function is neither regulated by endogenous MDM2 nor by Ser-20 phosphorylation in HCT116 cells.

A trivial explanation for MDM2 independence of the N-terminal mutants would be that the mutations in p53 destroy the MDM2 binding site, but we can rule this out, because Ashcroft et al. (18) showed that all of the mutants used in our study can bind to MDM2. Phosphorylation of Thr-18, which is predicted on structural evidence to block MDM2 binding, is unlikely to explain the absence of MDM2 regulation in HCT116 cells, because Thr-18 is not phosphorylated following DNA damage in these cells (19). Another explanation could be that MDM2 function is inhibited by p14ARF in HCT116 cells, but this is unlikely because one allele of the p14ARF gene is methylated and the other allele contains a codon 33 frameshift mutation (51). Finally, it is important to note that our results only demonstrate a lack of regulation by endogenous MDM2. It is quite possible that higher levels of MDM2 expression following transfection of MDM2 vectors would destabilize p53. Indeed, we previously noted that p53 stability is not responsive to changes in the level of endogenous MDM2 in the tumor cell lines where exogenous MDM2 can target p53 for degradation (25).

If MDM2 does not mediate the stabilization of p53 in response to DNA damage in HCT116 cells, there must be another mechanism. Because the 175H and 22Q/23S mutants were not stabilized, it is likely that a p53 target gene is involved in this process. It was recently reported that Pirh2 is a p53-inducible gene that targets p53 for destruction (41). RNAi against Pirh2 failed to stabilize wild type p53 in our study, indicating that Pirh2 is not required for p53 destabilization in untreated HCT116 cells. Two other mechanisms for regulating p53 stability have been described. p53 binding to the signalosome subunit COP9/Jab1 leads to p53 phosphorylation on Thr-150 and nearby residues followed by ubiquitination and proteasome-mediated degradation (52). p53 phosphorylation on Thr-81 by Jun N-terminal kinase (JNK) stabilizes p53, particularly in G0/G1 (53). It is possible that these mechanisms regulate p53 stability in HCT116 cells.

p53 phosphorylation on N-terminal serines increases binding to the transcription coactivator p300 (12) and promotes acetylation of lysines at the p53 C terminus (14). Consistent with these reports, we could see slight reductions in p21 and MDM2 expression after doxorubicin treatment in cells expressing the 15A, 20A, N6A, and 382R mutants. There were no convincing changes in p21 and MDM2 expression with the different mutants after 5FU treatment, although we could see
differences in apoptosis induction, particularly with the N6A mutant. Dissociation of transcription and apoptosis by the N6A mutant has also been reported in H1299 cells, in which exogenous p53 is constitutively active (54). We therefore tested induction of a panel of target genes by this mutant at the RNA level. The genes were selected on the basis of preliminary microarray screens for genes that respond to DNA damage in a p53-dependent manner, plus likely candidates culled from the literature. The differences in target gene induction by wild-type p53 and N6A were all small. The gene that showed the largest change was PUMA, which is incidentally the gene considered most likely to mediate apoptosis induction by p53 in these cells (35, 55). The simplest interpretation of our results is that most likely coactivators are required at the PUMA promoter, and the recruitment of these coactivators requires phosphorylation of Ser-15 and Ser-20. Other possible explanations for the reduced apoptosis induction by the mutant are that there is a difference in apoptosis induction, particularly with the N6A mutant, and a failure of the lentiviral system to recapitulate all perturbations in effector pathways during derivation of the knockout cells, or a failure of the lentiviral system to recapitulate all p53 stability.

Phosphorylation and acetylation of p53 at multiple sites has been documented in numerous studies (reviewed by Anderson and Appella, 11). Unlike many other cell types, the MDM2 feedback model can not explain regulation of p53 stability in these cells, which is incidentally the gene considered to be primary among the set of genes involved in apoptosis induction, including PUMA, PUMA, and MDM2. Unlike many other cell types, the MDM2 feedback model can not explain regulation of p53 stability in these cells, which is incidentally the gene considered to be primary among the set of genes involved in apoptosis induction, including PUMA, PUMA, and MDM2.

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