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The Human MDM2 Oncoprotein Increases the Transcriptional Activity and the Protein Level of the p53 Homolog p63*

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Genetic alteration of the p53 tumor suppressor gene, which monitors DNA damage and operates cell cycle checkpoints, is a major factor in the development of human malignancies. The p53 protein belongs to a family that also includes two structurally related proteins, p63 and p73. Although all three proteins share similar transcriptional functions and antiproliferative effects, each of them appears to play a distinct role in development and tumor suppression. One of the principal regulators of p53 activity is the MDM2 protein. The interaction of MDM2 with p53 inhibits p53 transcriptional activity and targets p53 for ubiquitin-dependent degradation. The ability of MDM2 to inhibit p53 functions is antagonized by the ARF oncosuppressor protein. We show here that like p53, the p63α and p63γ isoforms are able to associate with human MDM2 (HDM2). Overexpression of HDM2 increased the steady-state level of intracellular p63 and enhanced its transcriptional activity. Both effects appeared to be counteracted by ARF coexpression. These data indicate that p63 can be activated by HDM2 under conditions in which p53 is inhibited. Therefore, HDM2 expression could support p63-specific transcriptional functions on a common set of genes, keeping interference by p53 at a minimum.

The p63 gene, which maps on the 3q27–28 region, is one of the members of the p53 gene family. Unlike p53, it shows a complex pattern of expression due to alternative splicing and promoter usage that results in multiple isoforms with different biological activities (1, 2). Initiation of transcription in exon 1 produces the TA isotypes, containing the evolutionarily conserved transactivation, DNA-binding, and oligomerization domains, whereas initiation in exon 3′ gives rise to the ΔN isotypes that lack the TA domain (3). p63 shows a remarkable structural similarity to p53 and to the related p73 protein: ~60% of the amino acids of the human p63 and p73 proteins in the region corresponding to the DNA-binding domain are identical to those of p53 (4).

In contrast with the ubiquitous expression of p53, p63 exhibits a rather tissue-specific distribution in that it is most detectable in the basal layer of stratified epithelia, including the epidermis, where the ΔNp63α isotype is predominantly expressed (3). However, it is still not known how the expression of different isoforms of p63 is regulated in different tissues and during development.

All three members of the p53 family share similar transcriptional functions, as p63 and p73 can also activate many of the p53 target genes, although with varying efficiency (5). Moreover, like p73, p63 is able to induce apoptosis and growth suppression in a manner similar to p53 (4).

Molecular alterations of p63 or p73 in human cancers appear to be rare; unlike p53-deficient mice, those lacking p73 or p63 show no increased susceptibility to spontaneous tumorigenesis (6, 7). Viral oncoproteins such as SV40 large T antigen, adenovirus E1B, and human papilloma virus E6, which bind and inactivate p53, do not target p73 and p63 (2, 8). Thus, it seems likely that p63 and p73 are not potent suppressors of abnormal proliferation.

Unlike p53, both p73 (6) and p63 appear to contribute to normal development. This is most dramatically illustrated by reports showing that p63-deficient mice have severe defects in limb and skin development (9). Moreover, heterozygous germline mutations in the p63 gene are the cause of ectrodactyly-ectodermal dysplasia-clefting (10) and ankyloblepharon-ectodermal dysplasia-clefting (11) syndromes in humans.

p53 is normally a short-lived protein. Regulation of the p53 protein occurs to a large extent through control of protein stability, and the MDM2 (murine double minute 2) protein has been shown to play a key role in targeting p53 for degradation (2). The ARF (alternative reading frame) protein, one of the alternative products of the *INK4a* locus, binds to the MDM2 protein, preventing MDM2-dependent p53 degradation and transcriptional silencing (12). Concerning the other members of the p53 family, it has recently been demonstrated that p73 also binds MDM2. MDM2 inhibits p73-dependent transcription by masking the p73 transactivation domain and/or disrupting the interaction of p73 with p300/CBP (cAMP-responsive element-binding protein-binding protein), but it is clearly not involved in the degradation of p73 (13). Here, we have investigated whether HDM2 (human homolog of murine double minute 2) and ARF are involved in the control of p63 functions. We have found that p63 is able to physically interact with HDM2. Overexpression of HDM2 increased the steady-state level of intracellular p63 and enhanced its transcriptional activity. Both effects were counteracted by ARF coexpression. Because of its opposite effects on p53 and p63 protein stability and transcriptional activity, MDM2 expression could support p63-specific transcriptional functions on a common set of genes, at the same time reducing interference by p53.

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EXPERIMENTAL PROCEDURES

Plasmids—The p63γ and p63α cDNAs were isolated from a human skeletal muscle cDNA library using a PCR-based technique and cloned into the *Bam*HI site of the pcDNA3-His expression vector (Invitrogen) to express them as Xpress epitope-tagged proteins. The amplification sequence consisted of 35 cycles of 98 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min, after starting with a denaturation step at 95 °C and ending with an extending step at 72 °C for 10 min. A common p63 forward primer (5′-CGGGATCCATGTCCCAGAGCACACAGACAAATG) and a p63α-specific (5′-GCGTAGTTTCTCCTCCCCCTCACTCCTAGGCG) or a p63γ-specific (5′-GGTTTGGCTAGTCACATGGTATCCCTAGGCG) reverse primer were employed to obtain p63α and p63γ, respectively. Wild-type p53 in pcDNA3 and the L22Q/W23S p53 mutant in the pCMV vector were from Dr. G. Del Sal (originally from Dr. A. J. Levine). The 1.8-kb fragment containing the wild-type p53 cDNA was retrieved by *Eco*RI digestion and ligated into the pcDNA3-His vector to express p53 as an Xpress epitope-tagged protein. The human *Mdm2* (*HDM2*) cDNA cloned into the bacterial pGEX4T3 expression vector was from Dr. S. Soddu (originally from Dr. D. George). The BP100-CAT reporter, containing two copies of the p53RE motif derived from the *HDM2* intronic promoter, was provided by Dr. G. Del Sal (originally from Dr. B. Vogelstein, Johns Hopkins University, Baltimore). Human *Mdm2* (*HDM2*) was from Dr. B. Vogelstein. The 2.4-kb fragment containing the $p21^{WAF}$ promoter was retrieved from the pGL3-p21 (pWWP) plasmid (14) and ligated into the *Hin*dIII site of the pCAT0 plasmid to obtain the WAF-CAT[1] reporter construct. The 680-bp *Sal*I-*Pst*I *ARF*-CAT reporter from the *ARF* promoter will be described elsewhere.[2] The *ARF* cDNA, previously described (15), was cloned into the pcDNA3.1-His mammalian expression vector (Invitrogen). The pEGFP-C1 expression vector (CLONTECH) containing DNA sequences for the enhanced green fluorescent protein was used for normalization of transfection efficiency.

Cell Culture and Transfection—Saos2, C33A, and COS-7 cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. For CAT assays, C33A cells (1×10^5) were seeded in 100-mm diameter dishes and transiently transfected (16 h later) using the calcium phosphate precipitation method. Equal amounts of expression plasmids for p53, p63γ, and p63α were cotransfected along with the $p21^{WAF}$-CAT, BP100-CAT, or *ARF* promoter reporter construct. The total amount of transfected DNA (20 μg) was kept constant using empty vector DNA when necessary. Cells were collected 30–48 h after transfection; equal quantities of proteins, determined by the Bradford method (Bio-Rad, Munchen, Germany), were assayed for CAT activity using 0.1 μCi of [^{14}C]chloramphenicol and 4 mM acetyl-CoA. Separated products were detected and quantitated by a PhosphorImager (Molecular Dynamics, Inc.) and ImageQuant software. The pCMV-βgal plasmid (1.5 μg) was used to normalize CAT values for transfection efficiency.

Saos2 cells (2.5×10^5) were seeded into six-well multiplates and transfected using LipofectAMINE 2000 (Invitrogen) under the conditions suggested by the manufacturer. At 48 h after transfection, equal quantities of proteins (determined by the Bradford method) were assayed for CAT activity. The amounts of MDM2 and ARF expression plasmids used in the transactivation experiments are indicated in the legends.

GST Fusion Protein Association Assay—The GST-HDM2 fusion protein cloned into bacterial pGEX4T3 was expressed in *Escherichia coli* under the inducible *lac* promoter and purified on a glutathione-Sepharose 12B column. Protein-protein association assay was conducted as follows. 20 μl of *in vitro* translated, ^{35}S-labeled p63γ, p63α, L22Q/W23S p53, or wild-type p53 (TNT, Promega) were incubated for 1 h at 4 °C with the GST-HDM2 fusion protein coupled to glutathione-Sepharose beads (200-μl total reaction volume). The mixtures were washed three times with 50 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0), 100 mM NaCl, 0.1% Nonidet P-40, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. The bound proteins were analyzed on an 8% SDS-polyacrylamide gel and detected by autoradiography.

Co-immunoprecipitation—Saos2 cells (three 60-mm dishes) were transfected with 2 μg of parental pcDNA3-His, p63α, and p63γ with or without 2 μg of HDM2 expression plasmid with LipofectAMINE 2000. Cells were harvested 24 h after transfection and lysed in immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, and 10 μg/ml trypsin inhibitor). Debris was removed by centrifugation. Lysates (0.5 mg of protein) were precleared with 30 μl of protein A-agarose (50% slurry) and then incubated for 1 h at 4 °C with 2 μg of polyclonal anti-p63 antibody (H-137, Santa Cruz Biotechnology). Fresh protein A beads (30 μl) were added and incubated overnight at 4 °C. The beads were loaded directly onto an SDS-polyacrylamide gel after two washes with immunoprecipitation buffer. The co-immunoprecipitated proteins were detected by Western blotting using anti-MDM2 (smp14, Santa Cruz Biotechnology) and anti-Xpress (Invitrogen) antibodies.

Analysis of Protein Levels and Half-life Determination—Saos2 and COS-7 cells (2.5×10^5 in six-well plates) were transfected using the LipofectAMINE 2000 reagent. The pEGFP-C1 expression vector was included in these experiments as a control of transfection efficiency. At 48 h post-transfection, the cells were harvested by scraping in 100 μl of immunoprecipitation buffer containing 0.5% deoxycholate and lysed by sonication. Expression levels of both the transfected p63 and ARF proteins were determined by Western blotting using the anti-Xpress antibodies. The identity of the p63 bands was confirmed using anti-p63 antibodies (N-18, Santa Cruz Biotechnology). The $p21^{WAF}$ protein was revealed using mouse anti-human p21 antibody 6B6 (Pierce). Human MDM2 (HDM2) was detected with antibody smp14. The p53 protein was detected with the anti-Xpress antibodies or monoclonal mouse anti-human p53 antibody Pab240 (Pierce) as indicated. Bands were visualized with an enhanced chemiluminescence system (Pierce). To compare the stability of p63α and p63γ, Saos2 cells expressing the indicated cDNAs were treated with cycloheximide (final concentration of 80 μg/ml) and harvested at the indicated time points. Cells were processed for Western blotting as described above. The 26 S proteasome inhibitor ALLN (50 μM; Sigma) was used. Tubulin was detected with an anti-tubulin antibody (C-20, Santa Cruz Biotechnology). For reverse transcriptase-PCR, 24 h after transfection, cells were collected, and total RNA was isolated using the Trizol LS reagent (Invitrogen) following the manufacturer's instructions. 500 μg of total RNA were reverse-transcribed using 200 units of Superscript II (Invitrogen) and PCR-amplified as described above. The 600-bp fragment of the human hypoxanthine phosphoribosyltransferase gene was amplified using the following primers: 5′-CCTGCTGGATTACATTAAAGCACTG and 5′-CCTGAAGTACTCATTATAGTCAAGG.

RESULTS

Comparison of Transcriptional Properties of p63γ and p63α in Mammalian Cell Lines—The p63γ and p63α cDNAs encode proteins of 448 and 641 amino acids, respectively (4). The longer α isoform possesses an extended C-terminal region of 187 residues; but the rest of the protein, with the exception of the last 40 residues of the γ isoform, is shared by the two protein isoforms. The C-terminal region of p63α includes a sterile α-motif that has been described as a putative protein-protein interaction domain (16). The three major domains of p53 (NH_2-terminal transactivation, DNA-binding, and oligomerization domains) are conserved in both the γ and α isoforms. We isolated the p63γ and p63α cDNAs by reverse transcriptase-PCR from a human skeletal muscle library and cloned them into the pcDNA3.1-His expression vector. Before assessing the effect of HDM2 on the transcriptional activity of both p63 isoforms, it was of interest to compare the transcriptional properties of the two p63 isotypes on two canonical p53-responsive promoters, $p21^{WAF}$ and *HDM2* (BP100-CAT).

C33A ($p53^{-/-}$) and Saos2 cell lines, which not only lack endogenous p53, but also exhibit low levels of p73 (17), were transfected with equal amounts of p63γ or p63α expression vector together with the CAT reporter plasmids. As a positive control, we also transfected a p53 expression vector. As shown in Fig. 1 (*A* and *B*), both p63 isoforms stimulated CAT activity, although a significant difference in efficiency was observed: p63α enhanced CAT expression driven from either promoter less strongly than p63γ in both cell lines.

Although the *ARF* promoter does not contain p53-binding elements, it is *trans*-repressed by p53 (18),[2] suggesting the existence of an autoregulatory feedback loop limiting the effect

[1] The abbreviations used are: CAT, chloramphenicol acetyltransferase; GST, glutathione *S*-transferase; ALLN, *N*-acetyl-leucyl-leucyl-norleucinal-CHO; GFP, green fluorescent protein.

[2] T. Parisi, A. Pollice, A. Di Cristofano, V. Calabrò, and G. LaMantia, submitted for publication.

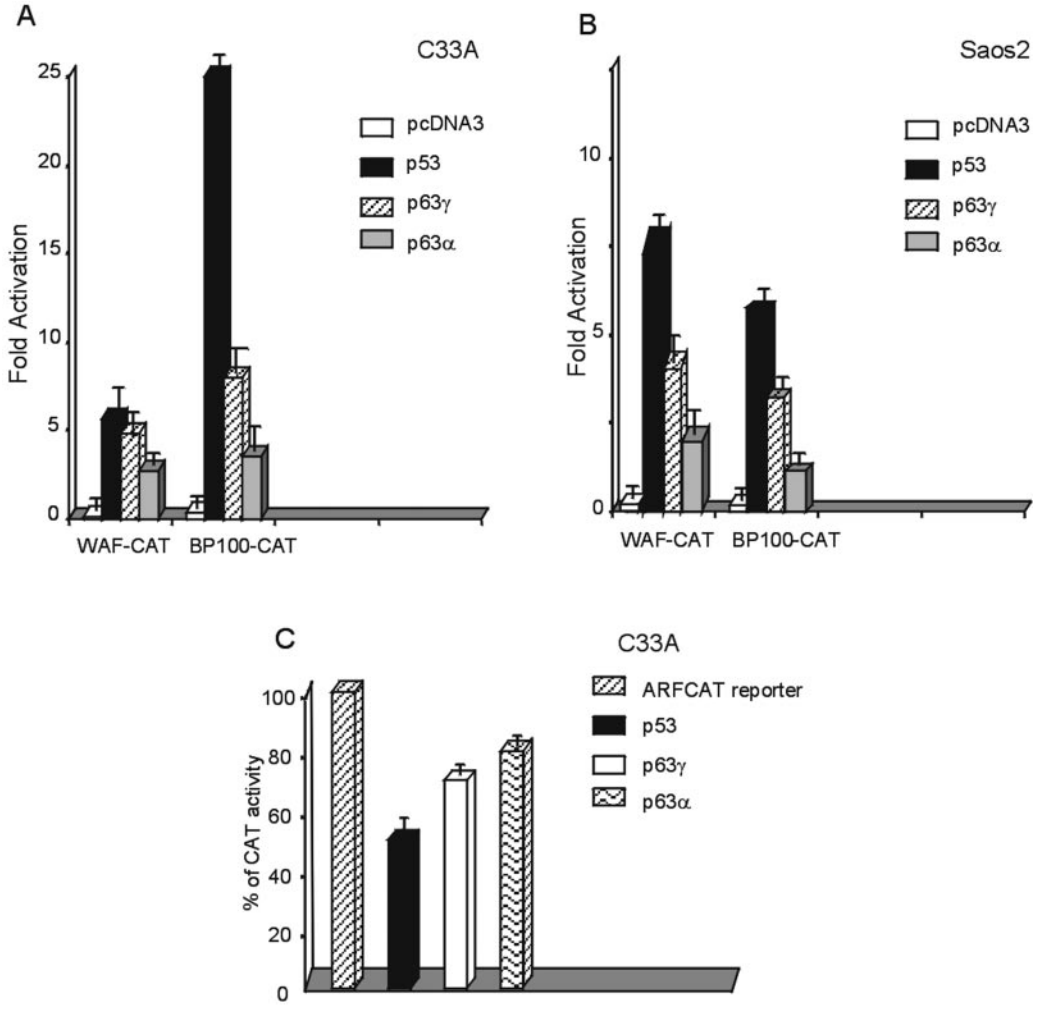

FIG. 1. **Transcriptional activity of p63 in mammalian cells.** *A*, C33A cells were transfected with the $p21^{WAF}$-CAT (1 μg) or BP100-CAT (1 μg) reporter plasmid. Expression plasmids for p53, p63γ, and p63α (10 μg each) were cotransfected. After 48 h, cells were harvested, and CAT was activity determined as described under "Experimental Procedures." The results of triplicate transfections are reported as the mean -fold activation with each of the effectors (activity with effector/activity with empty expression vector). The values presented were normalized with an internal control as described under "Experimental Procedures." S.D. values are shown by *error bars*. *B*, Saos2 cells were transfected with the $p21^{WAF}$-CAT or BP100-CAT reporter plasmid (0.2 μg). The expression vectors indicated (0.2 μg each) were cotransfected. Transfection was performed as described under "Experimental Procedures." The results of triplicate transfections are reported as the mean -fold activation with each of the effectors (activity with effector/activity with empty expression vector). The values presented were normalized with an internal control as described under "Experimental Procedures." S.D. values are shown by *error bars*. *C*, C33A cells were transfected with 10 μg of *ARF*-CAT reporter construct in combination with 10 μg of the indicated expression vectors. The CAT assay was performed as described under "Experimental Procedures." The basal activity of the *ARF*-CAT reporter was set to 100%. Values represent the means ± S.D. of three independent experiments.

of ARF on p53 stabilization. Based on the functional similarity between p53 and p63, we tested whether or not p63 was also able to inhibit transcription driven by the human *ARF* promoter. C33A cells were cotransfected with the *ARF*-CAT reporter plasmid and the p53, p63γ, or p63α expression plasmid. Fig. 1*C* shows that both p63γ and p63α reduced *ARF*-CAT expression, although less efficiently than p53. These results indicate that p63 also shares the *trans*-repression ability with p53. Again, p63γ appears to be more efficient than the α isoform.

To investigate whether the differences observed in the transcriptional properties of p63γ and p63α were due to different expression levels of the α and γ isoforms, we measured the protein levels of the two isotypes 48 h after transfection in the Saos2 cell line. The stronger transcriptional activity of p63γ cannot be attributed to a higher expression level, as Western blot analysis revealed that p63α levels exceeded those of p63γ (Fig. 2*A*). Similar results were obtained in COS-7 cells (data not shown). These results suggest that the extended C-terminal region, which distinguishes p63α from p63γ, could influence the level of the p63 protein, perhaps altering its half-life. We assessed this possibility by introducing expression vectors for p63α and p63γ into Saos2 cells and following their protein levels after treatment with cycloheximide (19). Because cycloheximide inhibits *de novo* protein synthesis, the half-life of the proteins could be determined by Western blot analysis in cells treated with the drug. As Fig. 2*B* clearly shows, p63α had a markedly prolonged half-life.

HDM2 Enhances the Transcriptional Activity of p63—Because MDM2 inhibited both p53 and p73 transcriptional activity, we wanted to determine whether it also affected p63-driven transcription. To investigate this point, we cotransfected Saos2 cells with the BP100-CAT reporter plasmid, p63γ (Fig. 3*A*), or p63α (Fig. 3*B*) as transactivator and increasing amounts of HDM2. As shown in Fig. 3 (*A* and *B*, *third* and *fourth bars*), cotransfection of p63γ or p63α and HDM2 expression plasmids in 1:2 and 1:4 molar ratios produced a remarkable enhancement of p63 transcriptional activity. As a control, we performed the same experiment using p53 as transactivator. As expected, coexpression of HDM2 considerably reduced the p53 transcrip-

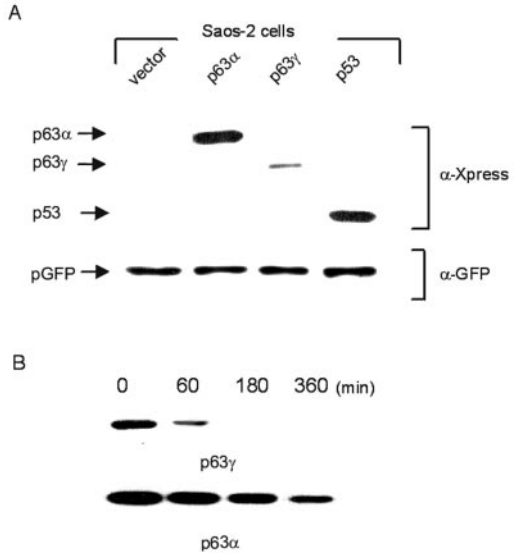

FIG. 2. **Levels of p63 isoforms.** *A*, Saos2 cells were transiently transfected either with 2 μg of empty vector (pcDNA) or expression plasmids (2 μg each) encoding p53, p63α, and p63γ. The pEGFP-1C vector (0.5 μg) was included as a control of transfection efficiency. Extracts from transfected cells were immunoblotted with anti-Xpress and anti-GFP antibodies as indicated. *B*, for measuring half-lives of p63α and p63γ, Saos2 cells were transfected with 2 μg of the indicated vectors. The cells were treated with cycloheximide (80 μg/ml) at 24 h post-transfection and harvested 0, 60, 180, and 360 min later. To obtain comparable starting levels of p63α and p63γ, 10 μg of extract from p63α-transfected cells and 50 μg of extract from p63γ-transfected cells were analyzed by Western blotting and detected with the anti-Xpress antibodies.

tional activity measured on the BP100-CAT reporter (Fig. 3*C*). A 1:1 molar ratio (*second bar*) of p53 to HDM2 was already sufficient to reduce the p53 transcriptional activity to 46% with respect to that observed without HDM2, and increasing amounts of HDM2 caused no more than an additional 10–16% reduction of p53 transcriptional activity.

In Vitro and in Vivo Association of p63 with HDM2—The interaction between MDM2 and p53 inhibits the p53 transactivation ability and targets p53 for ubiquitin-dependent degradation. Taking into account the effect of HDM2 on p63 transactivation ability, we decided to assess the effect of HDM2 expression on the level of the p63 protein. First, we wanted to test whether p63 may physically interact with HDM2. Extensive mutational analyses of the HDM2-binding domain of p53 (FSDLW) have identified Phe^{19}, Leu^{22}, and Trp^{23} as the critical residues for transcriptional activation and p53 binding by MDM2. We observed that these amino acid residues, except for a conservative Leu^{22}-to-Ile substitution, are present in the p63 MDM2-binding domain (FQHIW). The p63γ and p63α proteins were synthesized and [^{35}S]Met-labeled by an *in vitro* transcription/translation assay. The L22Q/W23S p53 mutant protein, which is unable to interact with MDM2 (20), and wild-type p53 were obtained in a similar way. SDS-PAGE followed by autoradiography revealed that proteins of the expected size and in comparable amounts were obtained in all cases (data not shown). The reticulocyte lysates were then incubated with the GSH-agarose-immobilized GST-HDM2 fusion protein. After appropriate washing, the bound proteins were subjected to SDS-PAGE and detected by autoradiography. Fig. 4*A* shows that the interaction of both p63γ and p63α with the GST-HDM2 protein was comparable to that of wild-type p53. Using the mutant p53 protein, a negligible amount of protein was detected.

To confirm the interaction between p63 and HDM2 in intact cells, we cotransfected Saos2 cells with p63α or p63γ and the

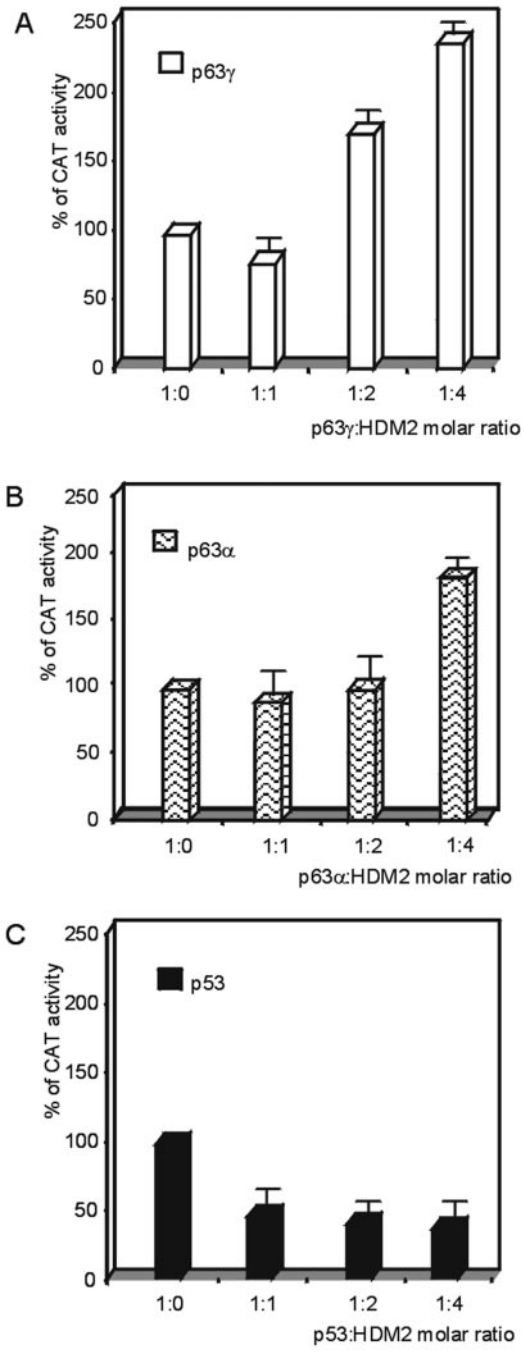

FIG. 3. **Transcriptional activity of p63α and p63γ in the presence of HDM2.** Saos2 cells were transfected with the BP100-CAT reporter plasmid (0.2 μg/dish) and expression plasmids for p63γ (0.2 μg; *A*), p63α (0.2 μg; *B*), and p53 (0.1 μg; *C*). Increasing amounts of HDM2 (corresponding to 1:1, 1:2, and 1:4 molar ratios of p63γ (*A*), p63α (*B*), and p53 (*C*) to the HDM2 expression vector) were cotransfected. The values obtained with p63γ, p63α, or p53 alone were set to 100%. All CAT activities measured in the presence of HDM2 are given as a percent of the values obtained with p53, p63γ, or p63α alone. Values represent the means ± S.D. of four independent experiments.

HDM2-encoding expression vector. Cellular lysates were immunoprecipitated with polyclonal anti-p63 antibody H-137 and probed with the monoclonal anti-MDM2 antibody. As shown in Fig. 4*B*, HDM2 was co-immunoprecipitated with the polyclonal anti-p63 antibody when coexpressed with p63α or p63γ.

HDM2 Increases p63 Protein Levels—Binding of MDM2 to p53 is required for targeting p53 for degradation (21); p73, however, binds to MDM2, but is refractory to MDM2-mediated

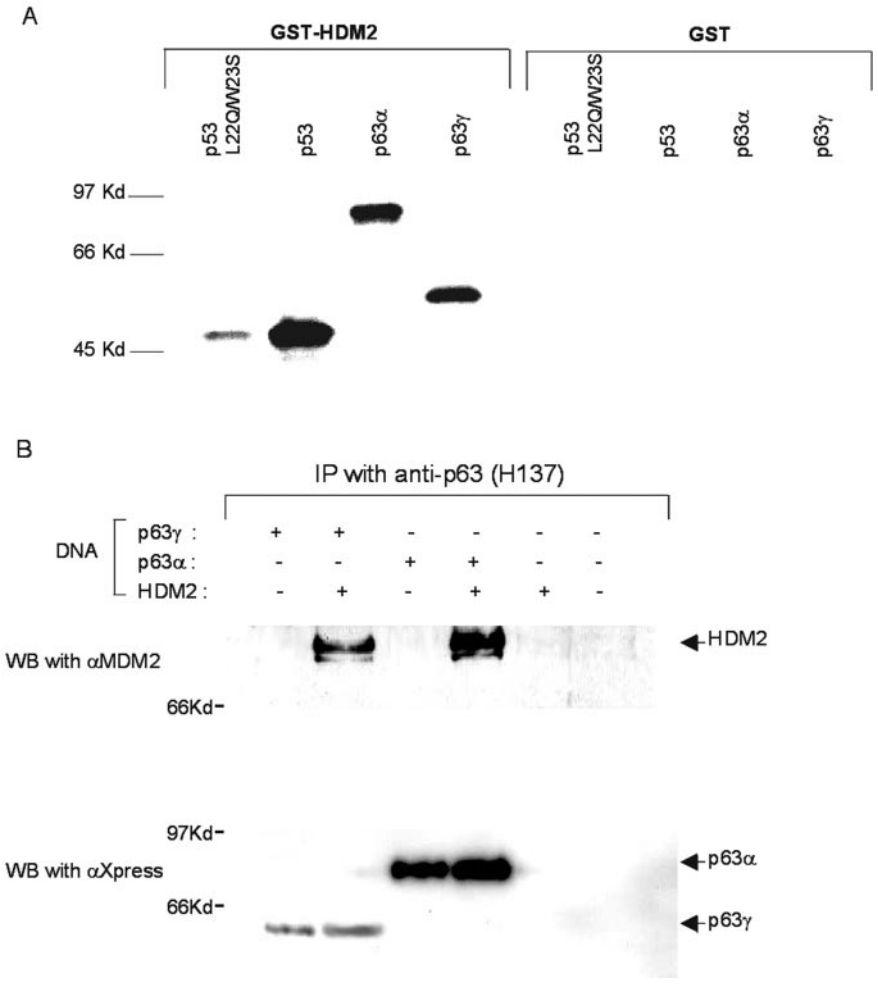

FIG. 4. **HDM2 binds to p63.** *A*, GST-HDM2 fusion proteins immobilized on Sepharose were incubated with 20 μl of *in vitro* translated, ^{35}S-labeled p53, L22Q/W23S p53, p63γ, or p63α at 4 °C for 1 h as indicated. Bound proteins were analyzed as described under "Experimental Procedures." A control of binding with GST alone is also shown. *B*, Saos2 cells were transfected with plasmid encoding p63γ or p63α with or without HDM2 as indicated. At 24 h post-transfection, cells were harvested for immunoprecipitation (*IP*). p63-HDM2 complexes were analyzed by immunoprecipitation using polyclonal anti-p63 antibodies. Proteins were revealed with anti-Xpress and anti-MDM2 antibodies (as indicated). The positions of molecular mass marker are indicated to the left. *WB*, Western blot.

degradation (13), indicating that binding to MDM2 is necessary but not sufficient for degradation. Recent findings indicate that a proline-rich sequence (from amino acid 92 to 112) of p53 is a degradation signal (19). This degradation signal is not present in the p73 and p63 proteins. To elucidate the effect of HDM2 on p63 protein levels, we transiently transfected COS-7 cells with equal amounts of p53 (Fig. 5*A*) and p63α (Fig. 5*B*) and increasing amounts of HDM2 expression vector. 0.5 μg of plasmid pEGFP-C1 were included as the transfection control. Cellular lysates were subjected to immunoblotting with antibodies against the Xpress epitope (Fig. 5*B*). The identity of the p63 bands was then confirmed using an antibody raised against the N terminus of the p63 protein (data not shown). The p53 protein was revealed with an antibody raised against an epitope corresponding to amino acids 156–214 of the human p53 protein (Fig. 5*A*), whereas the HDM2 protein was revealed with an antibody raised against an epitope corresponding to amino acids 154–167 of the human MDM2 protein (Fig. 5, *A* and *B*). Anti-GFP immunoblotting demonstrated that, in all cases, comparable transfection efficiency was achieved. HDM2-induced degradation of the p53 protein was detected easily, and the effect was found to be dose-dependent (Fig. 5*A*). Moreover, as the COS-7 cells expressed detectable levels of the endogenous p53 protein (Fig. 5*A*, *lanes 1–3*), a reduction of the endogenous p53 protein level was seen when HDM2 was overexpressed. In contrast, overexpression of HDM2 resulted in a remarkable increase in the level of the p63α protein (Fig. 5*B*). Moreover, a parallel increase in the level of endogenous p21^{WAF} was observed (Fig. 5*B*). Because we observed an effect of HDM2 on the p63 transcriptional activity in Saos2 cells, we performed similar experiments in this cell line with both p63 isoforms; and again, we noticed a progressive rise in the level of the p63α protein when increasing amounts of HDM2 were cotransfected (Fig. 5*C*).

To determine whether the elevation in the p63α protein level was due to an increase in transcription or stability of p63α mRNA, we performed reverse transcriptase-PCR experiments. Fig. 6*A* shows that the relative level of p63α mRNA was similar in the presence and absence of HDM2, suggesting that the effect of MDM2 on p63 may be post-translational. Fig. 6*B* shows that, after treatment with ALLN, a proteasome inhibitor, more p63α protein was detected, suggesting that the p63α protein may be degraded by a proteasome-dependent pathway. Significantly, ALLN did not further increase the level of p63α in the presence of HDM2, suggesting that HDM2 and ALLN may both act to prevent proteasome-dependent degradation. Similar results were obtained with the p63γ isotype (data not shown). Hence, HDM2 increases p63 protein levels under conditions in which p53 is degraded.

ARF Abolishes p63 Stabilization and Transcriptional Activation Induced by HDM2—Among the growing number of proteins that interact with MDM2, particular interest has recently been focused on ARF, which is encoded by the *INK4a* locus. Because ARF binds to the MDM2 protein, preventing MDM2-dependent p53 degradation and transcriptional silencing (12, 22), we predicted that ARF could counteract the effect of MDM2 on p63. To test this hypothesis, we cotransfected, in Saos2 cells, the BP100-CAT (Fig. 7*A*) or *WAF*-CAT (data not shown) reporter with a fixed amount of HDM2 and increasing amounts of ARF. We used a molar ratio (4:1) of HDM2 to p63α

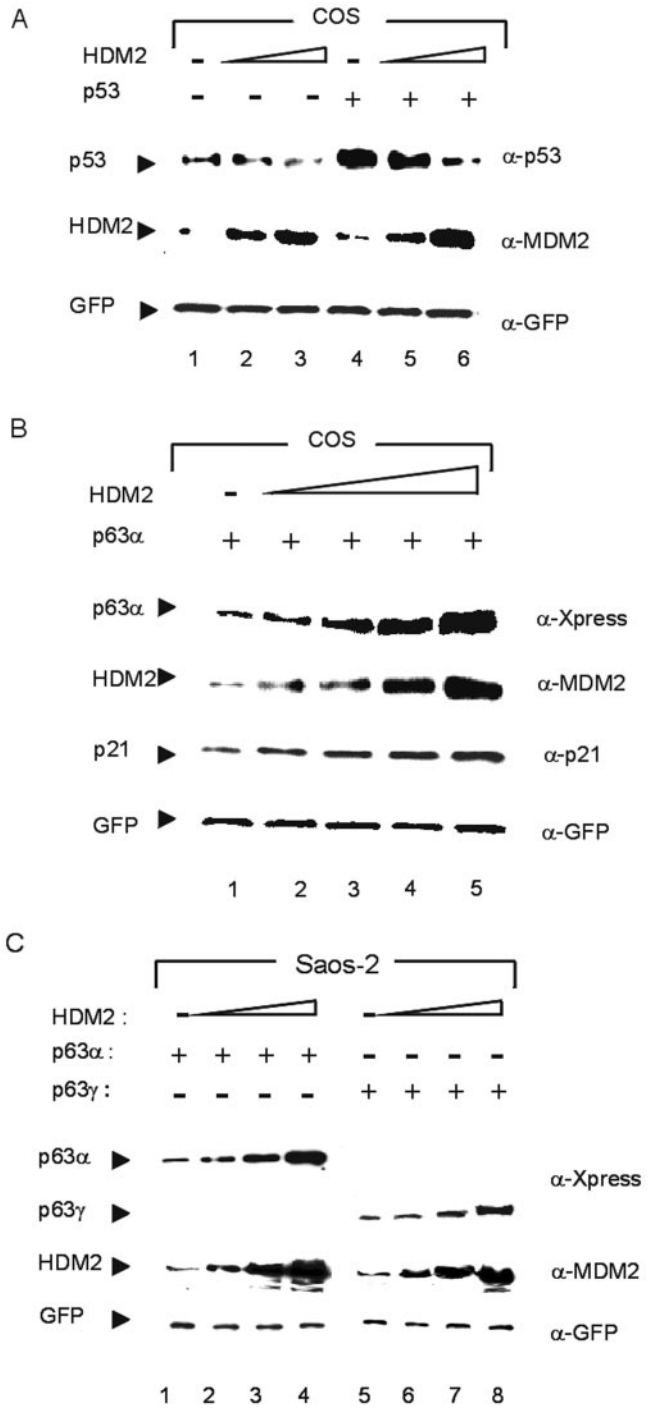

FIG. 5. **HDM2 increases p63 intracellular levels.** *A*, COS-7 cells were transfected with the pcDNA3 or p53 expression vector (0.2 μg/dish) in combination with increasing amounts of HDM2: 0.2 μg (*lanes 2* and *5*) and 1.6 μg (*lanes 3* and *6*). Extracts from COS-7 cells were subjected to immunoblotting with antibodies against p53, MDM2, and GFP as indicated. *B*, COS-7 cells were transfected with the p63α expression plasmid (0.2 μg/dish; *lanes 1–5*) in combination with increasing amounts of HDM2: 0.2 μg (*lane 2*), 0.4 μg (*lane 3*), 0.8 μg (*lane 4*), and 1.6 μg (*lane 5*). Extracts from COS-7 cells were subjected to immunoblotting with antibodies against the Xpress epitope, MDM2, $p21^{WAF}$, and GFP as indicated. *C*, Saos2 cells were transfected with expression plasmids (total of 2 μg of DNA) for p63α (0.2 μg/dish; *lanes 1–4*) and p63γ (0.2 μg/dish; *lanes 5–8*) in combination with increasing amounts of HDM2: 0.2 μg (*lane 2* and *6*), 0.4 μg (*lanes 3* and *7*), and 0.8 μg (*lanes 4* and *8*). At 48 h post-transfection, the cells were harvested and extracted as described under "Experimental Procedures." Western blotting was performed with anti-Xpress, anti-MDM2, and anti-GFP antibodies as indicated. The pEGFP-1C vector was included as a control of transfection efficiency.

that we know results in a strong enhancement of p63 transcriptional activation and protein stabilization (Figs. 3*B*, *fourth bar*; and 5*C*, *lane 4*). As shown in Fig. 7*A*, when increasing amounts of ARF were added, the increase in p63 transcriptional activity induced by HDM2 was progressively abolished. No effect was observed on the p63-driven transcription of the reporter plasmids when only ARF was expressed (Fig. 7*A*, *sixth bar*). We then tested whether or not ARF was also able to reduce the HDM2-induced enhancement of the p63 protein level. The p63α expression plasmid was transfected in the Saos2 cell line with or without a fixed amount of HDM2 expression vector and increasing amounts of ARF vector (Fig. 7*B*). Exogenous expression of HDM2 produced an increase in the p63 level (Fig. 7*B*, *lane 5*) that was progressively abolished by the addition of increasing amounts of ARF expression vector (*lanes 6–8*). Moreover, as the Saos2 cells expressed detectable levels of endogenous HDM2 proteins (Fig. 7*B*, *lane 1*), a slight reduction of the p63 protein level was also seen when ARF alone was overexpressed (*lanes 2–4*). Similar results were obtained using the p63γ expression vector (data not shown).

DISCUSSION

Although there exists extensive information on the relationship between MDM2 and p53, far less is known about a possible functional interaction of MDM2 with p63. In this study, we report that MDM2 overexpression causes an increase in overall p63 protein levels due, at least in part, to a reduced rate of p63 protein degradation. Moreover, although MDM2 represses p53 transcriptional activity, enforced MDM2 expression causes instead a considerable enhancement of p63-mediated transcription, which can be ascribed to the increase in transcriptionally active p63 protein.

While this paper was in preparation, several studies were published reporting conflicting results on the functional relationship between p63 and the MDM2 protein. For instance, it was reported that exogenously expressed MDM2 represses p63-mediated transcription (23). On the other hand, it was proposed that MDM2 is unable to affect its half-life or its transcriptional function (24), in conflict with the present and above-mentioned papers. It is well documented that transiently transfected p63 is able to strongly induce the endogenous MDM2 protein (25), and we repeatedly observed that only at a low level of p63 exogenous expression is the induction of endogenous MDM2 negligible, so that the stabilization effect by transfected MDM2 becomes apparent. However, compared with our results, both Kadakia *et al.* (23) and Little and Jochemsen (24) obtained remarkably higher levels of p63α and p63γ exogenous expression, already sufficient to induce expression of endogenous MDM2. A possible explanation of the apparent discrepancy with our results is that, under their experimental conditions, p63 exogenous protein had already undergone stabilization, so that addition of exogenous MDM2 caused no further effect.

Moreover, we also demonstrate that p63 proteins are able to form a complex with HDM2 both *in vitro* and in mammalian cells, suggesting that the mechanism through which HDM2 regulates p63 expression requires a physical interaction between these proteins. Because it is well established that ARF stabilizes p53 by binding and sequestering MDM2, we expected ARF to exert an inhibitory effect on p63 protein stabilization. In fact, ARF coexpression abolishes both MDM2-induced p63 protein stabilization and transcriptional activation, giving further evidence that p63-MDM2 interaction has a functional role.

A recent analysis of the molecular interactions of p63 in a yeast two-hybrid system (26) suggested that p63 does not associate with MDM2 family proteins. However, this analysis was performed using only the N-terminal portion of the p63

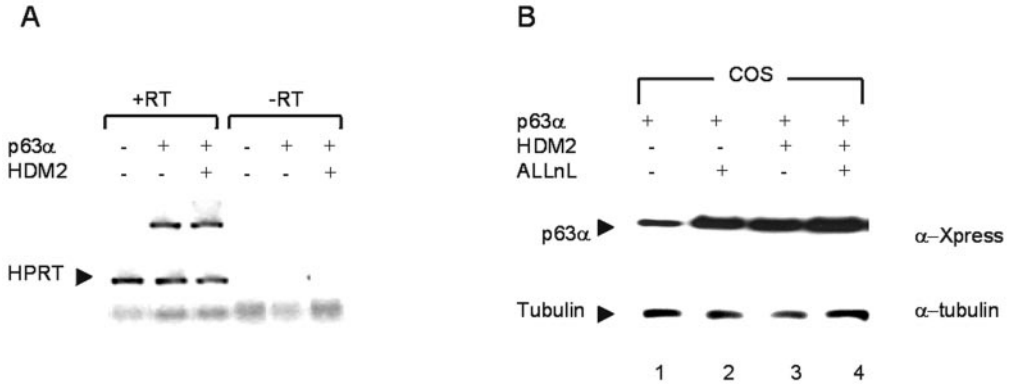

FIG. 6. **Regulation of p63 expression by HDM2.** *A*, COS-7 cells were transfected with p63α (0.5 μg) and empty vector or HDM2 (2 μg). The relative amount of p63α mRNA was analyzed by reverse transcriptase (*RT*)-PCR using p63 forward and reverse primers. *HPTR*, reverse transcriptase-PCR products obtained using oligonucleotides of the hypoxanthine phosphoribosyltransferase (*HPTR*) gene. *B*, COS-7 cells were transfected with p63α (0.5 μg) and empty vector or HDM2 (2 μg). After transfection, the cells were divided into two identical plates (vector (*lanes 1* and *2*) and HDM2 (*lanes 3* and *4*)) and allowed to grow for 24 h. Buffer (*lanes 1* and *3*) or ALLN (*ALLnL*; 50 μM; *lanes 2* and *4*) was added to the medium, and the cells were incubated for another 12 h. Cell extracts were subjected to immunoblotting for Xpress or tubulin.

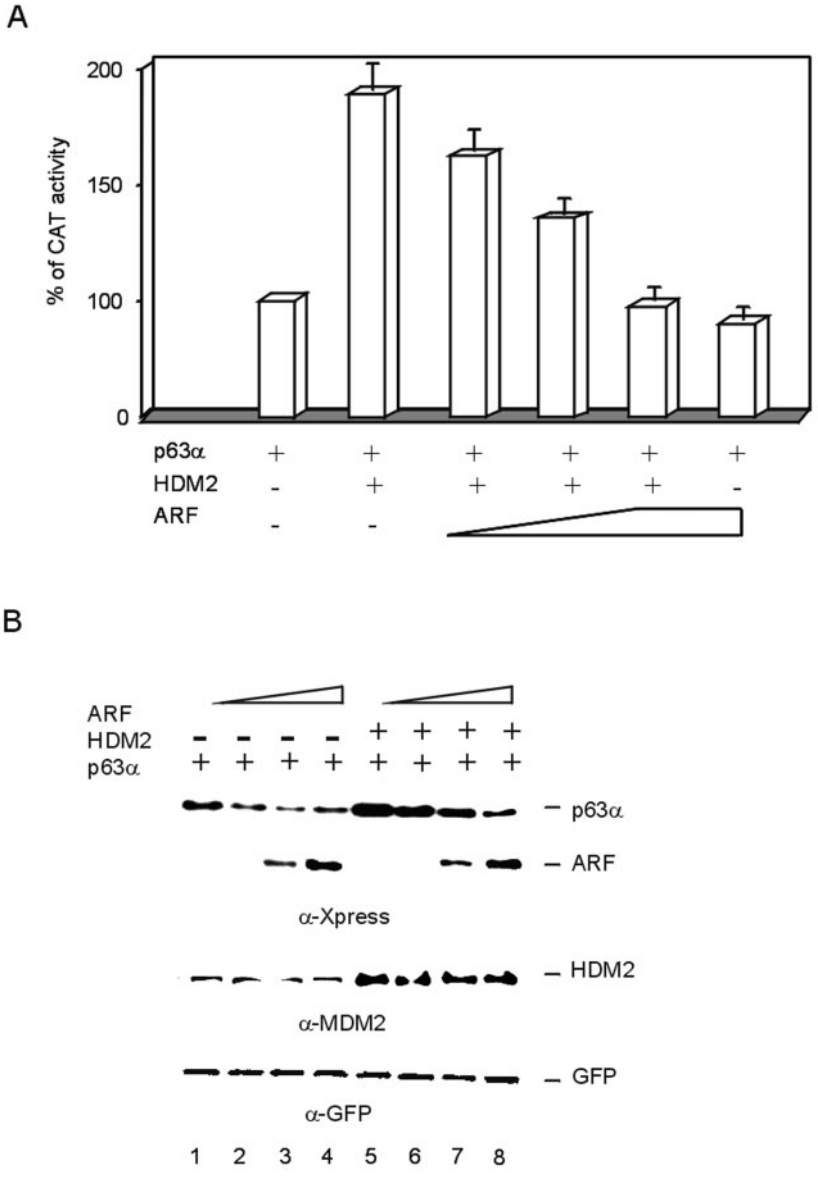

FIG. 7. **ARF counteracts HDM2-mediated p63 protein stabilization and transcriptional activation.** *A*, Saos2 cells were transfected with the indicated combinations of the following expression plasmids (total of 2 μg of DNA): BP100-CAT (0.2 μg/dish; *first* through *sixth bars*), p63α (0.2 μg/dish; *first* through *sixth bars*), HDM2 (0.8 μg/dish; *second* through *fifth bars*), and/or ARF (0.2 μg/dish (*third bar*), 0.4 μg/dish (*fourth bar*), and 0.8 μg/dish (*fifth* and *sixth bars*). CAT activities measured with p63α and HDM2; p63α and ARF; and p63α, HDM2, and ARF are given as a percent of the values obtained with p63α alone. Values represent the means ± S.D. of three independent experiments. *B*, Saos2 cells were transfected with expression plasmids (total of 2 μg of DNA/dish) for p63α (0.2 μg/dish; *lanes 1–8*) and HDM2 (0.8 μg/dish; *lanes 5–8*) and with increasing amounts of ARF expression plasmid: 0.2 μg (*lanes 2* and *6*), 0.4 μg (*lanes 3* and *7*), and 0.8 μg (*lanes 4* and *8*). Western blot analysis of cellular lysates were carried out with the indicated antibodies. The pEGFP-1C vector was included as a control of transfection efficiency.

protein (amino acids 1–111), so it cannot be excluded that other regions of the protein are essential for the p63-MDM2 interaction or that some tertiary structure formed by a more extended region may also be required for the binding. On the other hand, Little and Jochemsen (24) detected a weak interaction by an *in vitro* assay, but this could not be confirmed by co-immunopre-

cipitation in mammalian cells. Because we observed that MDM2 displays a lower affinity for p63 than it does for p53, it is possible that the use of less efficient antibodies or more stringent washing conditions could have hampered the analysis of the protein-protein interaction by immunoprecipitation. Remarkably, MDM2 has been reported to facilitate p63 export from the nucleus (23), but whether a direct MDM2 association with p63 is required for this activity remains to be elucidated.

Until recently, a similarly controversial question has been how MDM2 regulates the stability and transcriptional activity of the third member of the p53 family, the p73 protein (27, 28). Now a clear picture is emerging: MDM2 interacts with p73, stabilizing and enhancing its growth-suppressive function (29). Moreover, in sharp contrast to p53, MDM2 induces p73 to form nuclear aggregates that colocalize with MDM2 (30). Furthermore, p73 levels are increased in MDM2-expressing cells (30). Both p63 and p73 therefore bind to MDM2, but are refractory to MDM2-mediated degradation, indicating that binding is necessary but not sufficient for degradation. These results are not surprising given that, although the N-terminal MDM2-binding motif of p53 is conserved in both p63 and p73 (31), p53 has a unique sequence element (amino acids 92–112) that functions as a signal for MDM2-mediated degradation (19). How this sequence of p53 functions as a degradation signal remains to be defined.

Although we have not determined the precise mechanism by which MDM2 increases p63 protein levels, our data argue that, like the proteasome inhibitor ALLN, MDM2 may act by preventing p63 proteasome-dependent degradation. In addition to MDM2, the p300/CBP protein has also been shown to play a role in allowing efficient p53 degradation. Surprisingly, loss of p300 activity results in an inability to stabilize p53 in response to DNA damage, indicating that there is a complex relationship between p300 and p53 stability (32). It has been proposed that the reason why p73 is refractory to MDM2 degradation might be related to the observation that, unlike p53, p73 is unable to bind both MDM2 and p300 simultaneously (13). A similar mechanism could also explain the p63 resistance to MDM2 degradation. We are currently investigating the relationship between p63 and the p300/CBP coactivator as well as the exact pathway through which MDM2 induces an increase in p63 intracellular levels.

In conclusion, MDM2 seems to regulate p53 and its homologs through completely opposite mechanisms, suggesting that both p73 and p63 could be involved in specific cellular defense mechanisms against the deregulated expression of MDM2. We can also speculate that, in cells expressing both p63 and p53 proteins, certain stimuli that up-regulate MDM2 can, at the same time, activate p63 functions by keeping p53 activity at a minimum, whereas oncogenic stimuli that induce the ARF protein can cause the opposite. Moreover, once activated, p63 might contribute to its activation by keeping the level of ARF transcription low. These considerations suggest that the role of p63 may not be as central as that of p53 in tumor suppression, although it cannot be excluded that p63 could provide a protection from cancer development in tissues expressing both p53 and p63 proteins.

The role of p73 and p63 during normal development, the identification of differentiation genes specifically activated by p63 and p73 but not by p53 (33), and the difference in the ability to transactivate p53 target genes all strongly support the notion that these proteins, although closely related, have differentiated distinct physiological functions. The difference observed in the mechanisms adopted by MDM2 to control their functions further supports this emerging view.

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The Human MDM2 Oncoprotein Increases the Transcriptional Activity and the Protein Level of the p53 Homolog p63

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