The histone acetyl transferase Tip60 (HTATIP) shares many properties with the tumor suppressor p53 (TP53). Both proteins are involved in the cellular response to DNA damage, are subjected to proteasomal digestion following Mdm2-mediated ubiquitination, and accumulate after UV irradiation. We found here that knock-down of Tip60 affects the p53-dependent response following actinomycin D treatment, most likely because it inhibits p21 (CDKN1A) accumulation. Moreover, Tip60 is required for p53 to activate the endogenous p21 promoter, suggesting that it functions as a p53 co-activator. However, we also found that knock-down of Tip60 increases the turnover rate of p53 under normal growth conditions. Tip60 interferes with Mdm2-mediated degradation of p53, probably because it affects its subcellular localization. Taken together, our results suggest that Tip60 plays a double role in the p53 pathway: under normal growth conditions, Tip60 contributes to maintain a basal pool of p53 by interfering with its degradation; following DNA damage, Tip60 functions as a p53 co-activator. That these two distinct roles are linked during the p53-dependent response is an attractive hypothesis.

Eukaryotes have a variety of pathways to ensure genomic integrity. Following DNA damage, a cellular response is induced, which leads to cell cycle arrest to complete DNA repair or, alternatively, to apoptosis. In mammalian cells, the appropriate response to DNA damage is in part controlled by the p53 tumor suppressor (TP53) (1). p53 is a sequence-specific transcription factor, whose activity is induced by DNA damage. In turn, it regulates transcription of a variety of genes involved in proliferation control or apoptosis. Although several genes repressed by p53 have been described, the primary function of p53 is to activate transcription of genes containing p53-binding sites in their promoters, such as the gene encoding the p21 cyclin/cdk inhibitor (CDKN1A). The products of these genes are mostly involved in cell cycle control, apoptosis, and DNA repair (2). The balance between pro-apoptotic and growth arrest-regulated genes activated by p53 is believed to control the choice between apoptosis or growth arrest. In the absence of p53, the cellular response to DNA damage is abnormal leading to the accumulation of mutations. Therefore, not surprisingly, the p53-encoding gene is frequently mutated in many human cancers (3).

In the absence of DNA damage, p53 activity is largely controlled by the Mdm2 proto-oncoprotein. Mdm2 binds directly to p53, and represses its activity by two independent mechanisms: First, it functions as an E3 ubiquitin ligase for p53 (4) and thereby induces p53 degradation via the proteasome (5, 6). Second, binding of Mdm2 to p53 blocks transcriptional activation by p53 (7, 8). In response to DNA damage, p53 is phosphorylated by the ATM/ATR chk1/chk2 pathway, leading to the dissociation of the p53/Mdm2 interaction and the subsequent activation of p53-responsive genes (2).

The cellular response to DNA damage also involves the histone acetyl transferase (HAT)1 Tip60 (HTATIP), since overexpression of a dominant negative HAT-defective Tip60 mutant decreases both DNA repair and apoptosis upon induction of DNA double strand breaks (9). Tip60 is a HAT of the MYST family, which mostly acetylates histone H4 (10). It is part of a multiprotein complex, the Tip60 complex, which contains, in addition to Tip60, a number of proteins including the Trrap mediator protein (9). The Tip60 complex is believed to be the bona fide enzyme, since it can acetylate nucleosomes, whereas purified Tip60 is rather inefficient on nucleosomes (9). Moreover, this complex may be the mammalian homologue of the yeast NuA4 complex, which also contains a HAT of the MYST family (11).

Acetylation of the N-terminal tails of nucleosomal histones largely correlates with activation of transcription (12). Not surprisingly, Tip60 was found to function as a transcriptional co-activator in overexpression experiments (13, 14). Furthermore, the presence of endogenous Tip60 on c-Myc or NFκB target genes correlates with their transcriptional activation (15, 16). Moreover, overexpression of a HAT-deficient dominant negative mutant of Tip60 delayed the appearance of histone H4 acetylation on Myc-targeted promoters (15). All these results are consistent with a model in which Tip60 participates in transcriptional activation of specific genes through local histone acetylation. However, it should be noted that, in some instances, Tip60 was found to function as a transcriptional co-repressor (17).

We recently reported that Tip60 is a substrate of the E3

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1 The abbreviations used are: HAT, histone acetyl transferase; HA, hemagglutinin; CMV, cytomegalovirus; RT, reverse transcription; PBS, phosphate-buffered saline; Act. D, actinomycin D; siRNA, small interfering RNAs; DHFR, dihydrofolate reductase; PML, promyelocytic leukemia.
FIG. 1. Knock-down of Tip60 affects the p53-dependent response. A, total cell extracts from HeLa cells stably expressing HA-Tip60 (9) transfected by the indicated siRNA were analyzed for Tip60 expression (by an anti-HA Western blot, upper panel) and for HDAC 1,2 expression (lower panel). B, U2OS cells were transfected, or not, using the indicated siRNAs. Act. D was added where indicated 18 h before cell harvest. The cell cycle distribution of cells was then calculated as described under “Experimental Procedures.” This experiment was performed twice with very similar results. C, total cell extracts from U2OS cells transfected using the indicated siRNAs were tested for the presence of p21 and HDAC1, 2 by Western blot. The star indicates a nonspecific band detected by the p21 Western blot. D, U2OS cells were transfected with the indicated siRNA, then total RNA was reverse-transcribed and cDNAs were subjected to a real time PCR analysis for Tip60, p21, and ribosomal phosphoprotein P0 mRNA expression. After standardization relative to P0 mRNA expression, results were standardized to 100 for the expression of Tip60 (left) or p21 (right) mRNA in cells transfected by the C1 siRNA. E, Same as in C, except that Act. D was added where indicated 15 h before cell harvest, and that we used a shorter exposure than in C in order to see the induction of p21 expression following actinomycin D treatment.
ubiquitin ligase Mdm2, suggesting that p53 and Tip60 are regulated in a similar manner. Moreover, Tip60 expression was increased upon UV irradiation of Jurkat cells (18), raising the possibility that Tip60 and p53 functionally cooperate in the DNA damage response. We found here that Tip60 expression is critical for a proper p53-dependent response to actinomycin D, certainly by allowing activation of an important p53-responsive gene, the gene encoding the p21 cyclin/cdk inhibitor. The importance of Tip60 is underlined by the fact that Tip60 plays two distinct roles in the p53-dependent response: first, it functions as a co-factor for p53 to activate the endogenous p21 promoter, and second, it has the capacity to inhibit Mdm2-induced ubiquitination and proteasomal degradation of p53.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Antibodies, and siRNAs—**Details of constructions will be provided upon request. The anti-Tip60 DT antibody has been described previously (18). The anti-HA antibodies were purchased from Roche Applied Science (12CA5) or Eurogentec (BacoB). The anti-MDM2 antibody (SMP14), the anti-p21 antibody (C9), the anti-p53 antibodies (FL393, and DO-1), and the anti-PML antibody (PG-M3) were all purchased from Santa Cruz Biotechnology. All secondary antibodies were purchased from Sigma, except for the AlexaFluor-conjugated anti-mouse antibody (BD Biosciences).

Tip1 and c1 (control 1 from Dharmacon) siRNAs were purchased from Dharmacon, whereas Tip2, p53, and c2 were from Eurogentec (Belgium). C2 is derived from the mouse vinculin sequence and does not recognize any human mRNA. The sequence of the top strands of the various siRNA were as follows: c1, CAUGUCAUGUCUGCAACUCC-dTdT; c2, GCCGAGAUAGCCACCAUCUC-dTdT; Tip1, AGCGAAGAG-GAGGUGGUGG-dTdT; Tip2, CGCUCCAGUAGUGUGUGAG-dTdT; p53, GACUCAGUAGUGGUACUA-dTdT.

**Cell Culture and Transfections—**U2OS cells, H1299 cells, and HeLa cells stably transfected by HA-Tip60 (kindly provided by V. Ogryzko) were cultured in Dulbecco’s modified Eagle’s medium supplemented with antibiotics and 10% fetal calf serum. For proteasome inhibition, cells were treated with 100 μM ALLN (Sigma) for 12 h. Actinomycin D treatment (5 nM) was performed for 15 h. Transfections with plasmids were performed by the calcium phosphate co-precipitation method or with Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen) (for H1299 cells). Cells were harvested 24 h following transfection. For the siRNA experiments, 106 cells were transfected with 4 μg of lipofectamine (Invitrogen), and 10 μl of the double-stranded RNA according to the manufacturer’s instructions. Cells were harvested 48 h following transfection. Luciferase and β-galactosidase activities were measured using a kit from Promega and from Tropix, respectively, according to the manufacturers’ instructions.

**Immunofluorescence Analysis—**For immunofluorescence staining, cells were washed in PBS supplemented with antibiotics and 10% fetal calf serum. For proteasome inhibition, cells were fixed in PBS supplemented with 4% paraformaldehyde for 15 min, and permeabilized in PBS supplemented with 0.5% Triton for 10 min. After a 30-min incubation in blocking buffer (PBS-BSA3%), cells were stained with primary antibodies for 2 h at room temperature, then incubated with the secondary fluorochrome-conjugated antibodies. After several washes, coverslips were mounted in Dabco-DAPI, and analyzed with a microscope (Leica).

**Cell Extracts, Glutathione S-transferase Pull-downs, and Immunoprecipitations—**For total cell extracts, cells were directly lysed in 1× Laemmli sample buffer and subjected to Western blot analysis. Cell extracts followed by immunoprecipitations were performed from transfected cells as described in Ref. 19. Briefly, cells were collected in 500 μl of lysis buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 0.4% Nonidet P-40, 10 mM MgCl2, 2.5 mM CaCl2) supplemented with protease inhibitors (Complete EDTA-free, Roche Applied Science) and DNaSe (Coger). After centrifugation, total cell extracts were diluted in 500 μl of dilution buffer (50 mM Tris, pH 8.0, 0.4% Nonidet P-40). After a pre-clearing step, immunoprecipitations were performed overnight using 1 μg of antibody and 10 μl of protein A/protein G beads (Sigma). Immunoprecipitates were then washed three times in washing buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.4% Nonidet P-40, 5 mM MgCl2) and analyzed by Western blotting.

**Analysis of Cell Cycle Distribution—**Cells were treated with 100 μM BrdUrd (10 mM). Cells were then trypsinized, fixed with ethanol, and incubated for 30 min in 4 N HCl, 0.5% Triton. Cells were then extensively washed in PBS supplemented with 1% bovine serum albumin, incubated for 1 h with an anti-BrdUrd antibody (BD Biosciences) and then 30 min with a fluorescein isothiocyanate-conjugated secondary antibody (Sigma). Cells were then stained with propidium iodide (BD Pharmingen) and analyzed by flow cytometry (BD Biosciences) on FL1 (anti-BrdUrd) and FL3 (propidium iodide) to determine their cell cycle distribution.

**RT Q-PCR Analysis—**Total RNA were extracted from 1 × 106 transfected U2OS cells using RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Elution was performed with 50 μl of H2O. First-strand cDNA was generated using 15 μg of total RNA in a standard reverse transcriptase reaction using a poly(T) oligonucleotide as a primer and SuperScript II reverse transcriptase (Invitrogen, Life Technologies, Inc.). The final concentration of cDNA was 0.5 μg/μl. 1.5 μg of cDNA were then subjected to real time PCR analysis. Real time PCR was performed using Sybr green I (Sigma) and Platinum Quantitative PCR Supermix-UDG (Invitrogen) on a ICyclerQTM (Bio-Rad) real-time PCR device. Each experiment included a standard curve. The annealing temperature were 60 °C (Tip60 cDNA), 60 °C (ribosomal phosphoprotein P0 cDNA), 62.5 °C (p21 cDNA). The sequences of the oligonucleotides were as follow: GACGACCGGCTGCGAATG, TGGCGAGGCAAGGCTTAC for p21 cDNA, CAGGACCGGCTTCGAATTCCAGGACAGGCGACTTAC and CGGGGCAAGGCGAATCGGAG for Tip60 cDNA; GGGCAACCTGGAAGTCACCCT and CCATCAGCACCAGCTTTC for control ribosomal phosphoprotein P0 cDNA. Samples were analyzed in triplicates.

**RESULTS**

***Tip60 Expression Is Important for the p53 Pathway—***We previously showed that Tip60 is co-regulated with p53, presumably because both proteins are targeted by Mdm2 (18). These data led us to investigate the role of Tip60 in the p53 pathway. We designed a short interfering RNA targeting human Tip60. Although the effects of anti-Tip60 short interfering RNA treatment are difficult to assess on endogenous Tip60 protein due to its very low expression level (data not shown), Fig. 1A shows that this siRNA efficiently decreased Tip60 protein levels in a cell line stably expressing HA-tagged Tip60 (9). Moreover, by reverse transcription followed by real time PCR, we found that transfection of this anti-Tip60 siRNA in p53-negative U2OS cells led to the specific decrease of Tip60 mRNA expression (see Fig. 1D for example), with an efficiency comparable to what has been described recently (20). We investigated the effect of Tip60 knock-down on the cell cycle arrest induced by actinomycin D in U2OS cells (Fig. 1B). We found that, as expected,
actinomycin D treatment (Act. D) induced an accumulation of cells in G1 (black bars) and G2/M (hatched bars), both in non-transfected cells and in cells transfected by a control siRNA (c2) (which does not affect HA-Tip60 expression (data not shown)). The amount of S phase cells (gray bars) decreased in parallel.

Transfection of U2OS cells with anti-Tip60 siRNA led to a slight but reproducible increase of G2/M cells in the absence of actinomycin D. Strikingly, upon actinomycin D treatment, a significant amount of cells transfected with the anti-Tip60 siRNAs were still actively replicating DNA (gray bar). Moreover, the percentage of G1 cells was far lower than in cells transfected by the control siRNAs, indicating that the G1 arrest was ablated in these cells. In contrast, these cells still accumulated in G2/M, suggesting that the G2/M checkpoint was still active. This result indicates that upon Tip60 knock-down, the G1 arrest is defective, which indicates that Tip60 is required for a proper p53-response to actinomycin D. Strikingly, this result is similar to what has been found by Berns et al. (20) following ionizing radiation, indicating that the role of Tip60 in the p53 response is not dependent on the DNA damage inducer.

Arrest of cells in G1 following DNA damage is largely due to activation by p53 of the p21 cyclin/cdk inhibitor expression. We thus investigated the effect of Tip60 knock-down on p21 expression by Western blot. We found that endogenous p21 expression, which is controlled by endogenous p53 in U2OS cells (21), was severely inhibited upon Tip60 knock-down both at the protein (Fig. 1C) and the mRNA (Fig. 1D) level. Interestingly, knock-down of either Tip60 or p53 had a similar effect on p21 mRNA expression (Fig. 1D). Moreover, we found that, upon actinomycin D treatment (Act. D), accumulation of p21 protein was greatly decreased in cells transfected by the Tip60 siRNA compared with control cells (Fig. 1E). Thus taken together, these results suggest that Tip60 expression is required for the p53-dependent response, by a mechanism involving, at least in part, p21 expression control.

Tip60 Expression Is Required for Activation of Endogenous p21 Expression by Exogenous p53—Since Tip60 is a HAT, we envisioned the possibility that it could function as a p53 co-activator. To test this possibility, we performed gene reporter assays using promoters driven by p53 binding sites in the presence or absence of exogenous p53. Upon transient transfection of such reporters in U2OS cells, we did not find any effect of either Tip60 overexpression or Tip60 knock-down (data not shown and Fig. 2, graph), indicating that using this experimental setting, Tip60 was not involved in p53 transcriptional activity. We reasoned that this lack of effect could be due

**Fig. 3.** Knock-down of Tip60 expression decreases p53 expression. A, total cell extracts from HeLa cells stably expressing HA-Tip60 (9) transfected by the indicated siRNA were analyzed for Tip60 expression by an anti-HA Western blot. B, total cell extracts from U2OS cells transfected using the indicated siRNAs were tested for the presence of p53 HDAC 1,2 by Western blot. The asterisk indicates a nonspecific band detected by the p53 Western blot. C, same as in B, except that U2OS cells were transfected in the presence or in the absence (lane 1) of the indicated siRNAs and that ALLN was added where indicated 12 h before cell harvest. D, same as in B, except that Act. D was added 24 h later.
results indicate that Tip60 expression can antagonize the negative effects of Mdm2 on p53 expression.

To demonstrate that Tip60 affects the stability of p53 in the presence of Mdm2, we used the DHFR-ubiquitin fusion protein system (22). This system takes advantage of the observation that, when a protein is expressed as a ubiquitin fusion protein, the N-terminal ubiquitin-containing part is co-translationally cleaved off by ubiquitin-specific proteases generating free ubiquitin (or in this case, DHFR-ubiquitin) and the protein of choice. Since the DHFR-ubiquitin moiety and the protein of choice (in this case p53 or Mdm2) are expressed as a fusion protein from the same transcript and, thus, have an identical transcription and translation rate, comparison of the relative transactivation of a co-transfected p53-dependent reporter vector (graph). Thus, Tip60 expression is required for p53 to activate the endogenous p21 promoter, in agreement with the hypothesis that Tip60 functions as a co-activator for p53.

Tip60 Knock-down Enhances p53 Degradation—When performing these experiments, we also observed that in cells transfected by the anti-Tip60 siRNAs, endogenous p53 levels were consistently decreased (although the effect is weak in the experiment shown in Fig. 2). Because this finding was unexpected, we first confirmed it using another anti-Tip60 siRNA. This siRNA also decreased Tip60 protein levels in a cell line stably expressing HA-tagged Tip60 (Fig. 3A). We found that Tip60 knock-down with either of the two anti-Tip60 siRNAs significantly and specifically decreased p53 protein levels compared with cells transfected by a control siRNA (Fig. 3B, upper panel). To rule out any nonspecific effects possibly leading to p53 stabilization by the control siRNA, we performed additional controls and found that the control siRNA (Fig. 3C, lane 4) did not induce accumulation of p53 compared with mock-transfected cells (lane 1), or cells transfected with another control siRNA (lane 2). The decrease in p53 levels upon Tip60 knock-down was most likely due to a destabilization of p53, since p53 mRNA levels were not affected by Tip60 knock-down (data not shown) and since Tip60 siRNA did not have any effect on p53 accumulation in the presence of a proteasome inhibitor (Fig. 3C, ALLN). Moreover, we found that the effect of Tip60 knock-down on p53 levels was observed only in untransfected cells, since upon actinomycin D treatment, it was no longer detectable (Fig. 3D, Act. D). Thus, taken together, these results indicate that endogenous Tip60 is involved in the control of p53 levels in normally growing cells by protecting p53 from proteasomal degradation.

Tip60 Interferes with Mdm2-induced Degradation of p53—P53 levels in noninduced cells are largely controlled by Mdm2, which functions as an E3 ubiquitin ligase for p53 thereby inducing its degradation through the proteasome. To test whether Tip60 is important for p53 expression by interfering with its Mdm2-induced degradation, we transfected U2OS cells with expression vectors for the various proteins, together with a CMV-luciferase reporter vector. After standardization according to luciferase activity for transfection efficiency and any nonspecific effect on the CMV promoter, total cell extracts were prepared by boiling cells directly in Laemmli sample buffer to ensure efficient extraction of all proteins. Then, the amount of exogenous p53 was determined by Western blot analysis. As expected, overexpression of Mdm2 led to a decrease in the steady state levels of transiently expressed p53, reflecting Mdm2-induced p53 degradation (Fig. 4A, lanes 1 and 2). Co-expression of Tip60 resulted in a dose-dependent restoration of p53 levels, although it had no effect on the expression of a co-expressed HA-tagged Ku80 used as a control. Importantly, the effect of Tip60 was not due to a decrease in exogenous Mdm2 expression. On the contrary, Mdm2 steady-state levels were also increased by exogenous Tip60 (Fig. 4A). Moreover, Tip60 expression had no effect on p53 levels in the absence of exogenous Mdm2 (data not shown). Thus, taken together, these
levels of p53 with those of DHFR-ubiquitin allows to unambiguously determine if Tip60 affects Mdm2-induced degradation of p53. Using this system, we found that Mdm2 decreased p53 stability, as expected (Fig. 4B). Co-expression of Tip60 restored normal p53 levels without affecting DHFR-ubiquitin levels, demonstrating that Tip60 interferes with Mdm2-mediated degradation of p53. In addition, a similar analysis for Mdm2 showed that Mdm2 was also stabilized by Tip60 expression (Fig. 4C).

Tip60 has been shown to physically interact with Mdm2 (18). To investigate whether Tip60 inhibited Mdm2-induced degradation of p53 through physical interaction, we used two deletion mutants of Tip60 (Fig. 4D). We found that Tip60 259–396 (M2), which contains the minimal Mdm2 binding domain, was able to stabilize p53 in the presence of Mdm2, as well as Mdm2 itself (Note that the mutant is less efficient in stabilizing p53 than full-length Tip60, presumably because it is less well-expressed (right panel)). On the contrary, Tip60 131–258 (M1), which does not bind Mdm2 (18), did not significantly affect p53 or Mdm2 levels. Thus, the reversal of Mdm2-induced p53 degradation correlates with the ability of Tip60 to bind to Mdm2.

**Tip60 Affects p53 and Mdm2 Subcellular Localization**

Tip60 is itself a substrate of Mdm2 ubiquitin ligase activity. Moreover, we did not find any effect of Tip60 on in vitro ubiquitination of p53 by Mdm2 (data not shown). Finally, Fig. 4B results show that ubiquitinated forms of p53 seem also to be stabilized by Tip60 expression. Taken together, these findings suggest that Tip60 interferes with a subsequent step of p53 degradation rather than ubiquitination. This step could be nuclear export, since, although there is recent evidence that p53 degradation can occur to some extend in the nucleus (23, 24), interfering with Mdm2 and p53 nuclear export clearly affects p53 degradation (25–27). We thus investigated the effect of Tip60 on p53 subcellular localization. Transfected p53 was always homogeneously nuclear (Fig. 5A, left panels). Co-expression of Mdm2 reduced p53 staining without affecting its localization (data not shown). Strikingly, in the presence of Mdm2 and Tip60, p53 appeared as dots in a significant proportion of transfected cells (about 50%) (right panels). This relocalization is likely to be mediated by physical recruitment of ectopically expressed p53 by exogenous Tip60, since when p53 staining appeared as dots, it always co-localized with Tip60 dots (Fig. 5B). Co-localization of Tip60 with PML indicates that these dots were actually PML bodies (Fig. 5C). Moreover, we found that exogenous Tip60 can also induce the relocalization of endogenous p53 to PML bodies (Fig. 5D). Taken together, these data indicate that Tip60 affects p53 localization, in agreement with the possibility that it interferes with p53 degradation by affecting its nuclear export.

**Ternary Complex Formation between p53, Tip60, and Mdm2**

Co-localization of p53 and Tip60 in PML bodies is only observed in the presence of Mdm2. Furthermore, Tip60 and
p53 bind to different domains of Mdm2 (18, 28). These data led us to hypothesize that Mdm2 could function as a bridge mediating a physical interaction between p53 and Tip60. To test this possibility, we performed co-immunoprecipitation experiments from transfected U2OS cells (Fig. 6A). We found that in the presence of expression vectors for p53, Tip60, and Mdm2, immunoprecipitation of p53 led to the co-immunoprecipitation of Tip60 (lane 2). This co-precipitation was specific, since Tip60 was not found in control immunoprecipitates (lane 4). However, in the absence of ectopically expressed Mdm2, co-precipitation of Tip60 with p53 was largely decreased (lane 1), although both proteins were nicely expressed (inputs, lower panel). These results indicate that p53 and Tip60 can physically associate in transfected cells in a Mdm2-dependent manner, in agreement with the possibility that Mdm2 functions as a bridge leading to the formation of a ternary complex. Furthermore, we found by immunofluorescence that exogenous (Fig. 6B) or endogenous Mdm2 (Fig. 6C) co-localized with Tip60 and p53 in PML bodies. Thus, taken together, these results suggest that Tip60 could form a ternary complex with Mdm2 and p53, thereby perhaps preventing them from being exported from the nucleus and subjected to proteasomal degradation.

FIG. 6. Ternary complex formation between Tip60, p53, and Mdm2. A, whole cell extracts from U2OS cells transiently transfected with 10 μg of pCMV p53 and 20 μg of pCDNA3 Tip60 in the presence or in the absence of 10 μg of pCMV Mdm2 were immunoprecipitated with 1 μg of anti-p53 antibody or 1 μg of anti-E2F-1 antibody (KH95) as a control (Irr.). Immunoprecipitated proteins were tested for the presence of transfected Tip60 by an anti-HA Western blot (upper panel). B, U2OS cells transiently transfected with 1 μg of pCMV p53, 1 μg of pCMV Mdm2, and 2 μg of pCMV 2N3T Tip60 were stained using an anti-Tip60 antibody and an anti-Mdm2 antibody. C, U2OS cells transiently transfected with 1 μg of pCMV p53 and 2 μg of pCMV 2N3T Tip60 were stained using an anti-Tip60 antibody and an anti-Mdm2 antibody.

DISCUSSION

Role of Tip60 in the p53 Pathway—In this article, we provide evidence that Tip60 expression is required for a complete p53-dependent response. Indeed, it is required for p21 expression and G1/S checkpoint activation following actinomycin D-induced DNA damage (Fig. 1). Interestingly, such a role of Tip60 was already demonstrated following ionizing radiations (20).
Role of Tip60 in the p53 Pathway

What could be the mechanism by which Tip60 plays its role in the p53 pathway? We found that, following Tip60 knock-down, exogenous p53 was no longer able to activate p21 expression (Fig. 2), consistent with the possibility that Tip60 functions as a co-activator for p53 transcriptional activity. Note, however, that we cannot rule out the possibility that the effect of Tip60 is indirect: for example, it could be required for the expression of a p53 co-activator. Whatever the mechanism, Tip60 is an important cofactor for p53. Interestingly, we previously showed that Tip60 expression increases following DNA damage, in that case UV irradiation (18). This increase could be important to adapt the levels of an important p53 co-factor with that of p53 itself.

In this article, we also demonstrate another more unexpected role of Tip60. We found that it was able to inhibit Mdm2-induced degradation of p53, thereby allowing a basal expression of p53 in unstressed cells. Our observation is likely to be relevant since we obtained similar results using two different siRNAs (Fig. 3) and we found that Tip60 protects p53 from Mdm2-induced degradation when overexpressed (Fig. 4). However, these results stand in contrast to what was found by Berns et al. (20) who did not find any decrease in p53 levels in cells stably expressing anti-Tip60 siRNAs. This discrepancy could be caused by a difference between transient inhibition of Tip60 expression (our study) or a stable inhibition (their study). Indeed, negative feedback loops (such as the Mdm2-dependent one) could restore normal levels of p53 in stably established cell lines. Alternatively, it is possible that a more efficient Tip60 inhibition should be achieved to detect an effect on p53 levels. Indeed, we consistently noticed that when the effect of Tip60 knock-down was not very strong, p53 basal levels were only weakly decreased (see for example Fig. 2).

Mechanism of Tip60-mediated p53 Stabilization—What could be the mechanism by which Tip60 inhibits Mdm2-induced p53 degradation? Because it is itself a substrate of Mdm2 (18) and because it does not significantly affect Mdm2 E3 ligase activity toward p53 in vitro (data not shown), Tip60 does not seem to function through the inactivation of Mdm2 enzymatic activity (e.g. by interfering with the interaction of Mdm2 with its cognate E2 ubiquitin-conjugating enzymes). We rather favor a mechanism in which Tip60 interferes with a subsequent step of Mdm2-induced p53 degradation. This step could be nuclear export of p53 and Mdm2, since nuclear export is required for p53 degradation (25–27).

Interestingly, such a mechanism has already been proposed for p53 stabilization by p19ARF (29). Consistent with this hypothesis, Tip60, when overexpressed, is able to change the subnuclear localization of endogenous Mdm2 and p53 (Figs. 5 and 6). Moreover, our result with Tip60 deletion mutants indicates that the effect of Tip60 involves the direct Tip60/Mdm2 interaction (Fig. 4).

Although we have not proven it directly, it is tempting to speculate that stabilization of p53 by Tip60 involves the formation of a ternary complex between p53, Mdm2, and Tip60. Indeed, all three proteins co-localize in PML bodies (Figs. 5 and 6). Formation of a ternary complex between endogenous proteins is difficult to demonstrate, because it requires immunodepletion of one of the three components of the ternary complex. However, endogenous proteins are likely to form also this ternary complex since p53 and Mdm2 on one hand and Tip60 and Mdm2 on the other hand are known to interact at endogenous levels. Interestingly, we found that Tip60, which is a target of Mdm2 ubiquitination (18), is also stabilized by p53 expression.2 Taken together, these results thus suggest that Mdm2 induces the degradation of both p53 and Tip60, except when the three proteins are embedded in a ternary complex.

A Function of the Ternary Tip60/p53/Mdm2 Complex in Transcription?—Our results also raise the intriguing possibility that a ternary Tip60/Mdm2/p53 complex could also be involved in transcriptional regulation, for example in allowing a rapid response to DNA damage or other kinds of stress. Indeed, since Mdm2 has been shown by chromatin immunoprecipitation to bind p53-responsive promoters in the presence of p53 (30), it could be envisioned that Tip60, and thereby its HAT activity, is recruited to p53-responsive promoters before stress. Interestingly, and in perfect agreement with this hypothesis, Espinosa et al. (31) found that p53 was bound to the p21 promoter and histone H4 was acetylated before promoter activation (31). It is thus tempting to speculate that the ternary complex we found here is bound to the p21 promoter, with Tip60 mediating histone acetylation and Mdm2 blocking a subsequent step of transcription activation.

Such a mechanism would allow a rapid transcriptional response to signals activating p53 (31). Indeed, most of such signals end up in the disruption of the p53-Mdm2 interaction. Promoter-bound p53 proteins would then be able to activate transcription immediately. Consistent with this idea, chromatic immunoprecipitation assay experiments ruled out the “latency model” for p53 DNA binding activity and demonstrated that p53 is perfectly able to bind to its target promoters in the absence of signaling (32). An obvious question in this context is what would happen to Tip60 following disruption of the Mdm2/p53 interaction. Since Tip60 is probably a co-activator of p53 on the p21 promoter (Fig. 2), it is tempting to speculate that it would remain bound to the promoter in an Mdm2-independent manner and participate in transcriptional activation of p53 responsive genes. For example, it is possible that following stress, p53 or Tip60 are post-translationally modified in a manner allowing Mdm2-independent interactions between the two proteins. Consistent with this possibility, p53 acetylation, which is induced by DNA damage, allows the recruitment of TRRAP, a component of the Tip60 complex (33).

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