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TIP60 Represses Transcriptional Activity of p73β via an MDM2-bridged Ternary Complex*[S]

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TIP60, a histone acetyl transferase, acts as a p53 coactivator by interfering with MDM2-mediated degradation of p53. However, little is known about its functional regulation of p73, which has structural features similar to p53. In this study we found that TIP60 represses apoptosis, which is induced by exogenous and endogenous p73β. TIP60 also negatively regulated the expression of p73β downstream target genes such as p21 and Bax. Moreover, the specific repression of p73β-mediated transactivation by TIP60 was independent of p53 expression and not due to histone deacetylase recruiting transcriptional machinery. Transcriptional activities of both p73 splicing variants, p73α and p73β, were also repressed by TIP60. Furthermore, TIP60 markedly enhanced p73β binding affinity to MDM2 and physically associated with MDM2 through its zinc finger domain, which is specifically localized in the nucleus. Therefore, we demonstrate that TIP60 forms a ternary complex with p73β, which is directly bridged by MDM2. It is important to note that our findings contribute to a functional linkage between TIP60 and p73β through MDM2 in the transcriptional regulation of cellular apoptosis.

p73 has significant sequence homology with p53, which provides similar structural features important for transcription factors (1, 2). Both p73 and p53 have an N-terminal transactivation domain, a central DNA binding domain, and a C-terminal oligomerization domain. Although p73 transcriptionally activates certain p53-responsive genes, which are closely related with cell cycle arrest and apoptotic responses (1), there are some functional differences between p73 and p53. For example, some viral oncoproteins including human *Papillomavirus* E6 and SV40 T-antigen do not interact with p73 even though they bind to p53 to inhibit its transcriptional activity (3, 4). The expression level of p73 is not affected by exposure to DNA-damaging agents such as ultraviolet irradiation, which efficiently increases p53 transcriptional activity (1). Furthermore, p73 was known not only to be necessary for survival and long-term maintenance of central nervous system neurons including postnatal cortical neurons (5) but also to have an anti-apoptotic role during developmental neuron death (6).

p73 is specifically activated by post-translational modifications including Chk1 phosphorylation (7), p300/CBP[3]-mediated acetylation (8), and PIAS1 sumoylation (9), which also affects p53 biological activities (10–12). Transcriptional activation of p73 is repressed by the association of MDM2 with p300/CBP, which is a transcriptional co-activator of p73 (13). Amphiphysin IIb-1 also inhibits p73 transactivation by sequestering nascent p73 proteins in the cytoplasm (14). Proteasome-dependent p73 degradation via ubiquitination is regulated by its interaction with Itch, which does not bind to p53 (15, 16). However, the exact underlying mechanism associated with p73 regulation is not clear.

TIP60 (HIV Tat-interacting protein, 60 kDa) was identified as an interaction partner for the HIV-1 Tat protein, which increases Tat transactivation of the HIV-1 promoter (17). The MYST (MOZ, Ybf2/Sas3, SAS2, and TIP60) domain defines TIP60 as part of the MYST family of histone acetyltransferase (HAT) proteins that are evolutionarily conserved from yeast to human (18). TIP60 functions as a transcriptional coactivator or corepressor depending upon the cellular context or the promoter site (19). For example, TIP60 associates with transcriptional activators such as HIV-1 Tat (17), type I nuclear hormone receptors (20, 21), and amyloid-β precursor protein) (22). In contrast, it has also been implicated in the negative regulation of gene expression through binding to the transcriptional factor CREB (cAMP response element-binding protein) or the transcriptional repressor ZEB (zinc finger E box-binding protein) (23, 24). However, the role of TIP60 HAT activity in these processes remains to be elucidated.

TIP60 was shown to act as a coactivator of p53-mediated transactivation. By interfering with MDM2-mediated p53 degradation through the formation of a ternary TIP60·MDM2·p53

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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[3] The abbreviations used are: CBP, cAMP-response element-binding protein (CREB)-binding protein; HIV, human immunodeficiency virus; TIP60, HIV Tat-interacting protein 60; hTIP60, human TIP60; HDAC, histone deacetylase; HAT, histone acetyltransferase; MDM2, mouse double minute 2; NA, 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone; PBS, phosphate-buffered saline; siRNA, small interfering RNA; shRNA, small hairpin RNA; WB, Western blot; FL, full-length; HA, hemagglutinin; HEK, human embryonic kidney; GFP, green fluorescent protein; ChIP, chromatin immunoprecipitation; CMV, cytomegalovirus.

complex (25), TIP60 increases p53 stability. The TIP60 transcriptional complex was also able to up-regulate p53-responsive genes including p21, GADD45 (growth arrest and DNA damage inducible gene 45), and MDM2 in response to DNA damage. Recently, it was also proposed that TIP60-dependent acetylation of p53 at Lys-120 plays a critical role for p53 activation to the apoptotic response (26, 27).

However, little is known about TIP60 functional regulation of p73 in cellular apoptotic responses. Therefore, we investigated the effects of TIP60 on p73 transcriptional activation using various human cell lines transiently transfected with a combination of knockdown or up-regulated expression plasmids of TIP60 and p73.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Human embryonic kidney 293 (HEK293), SK-OV-3, H1299, and HeLa cells were obtained from the American Type Culture Collection, Manassas, VA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen) and penicillin-streptomycin (50 units/ml). SK-OV-3 and H1299 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. 2,3-Dichloro-5,8-dihydroxy-1,4-naphthoquinone (NA) was provided by Dr. S. W. Ham at the Department of Chemistry at Chung-Ang University and dissolved in DMSO. Trichostatin A was purchased from Sigma-Aldrich. Transient transfection was performed by Lipofectamine 2000 (Invitrogen) with different plasmid DNA according to the manufacturer's instructions.

Plasmids—FLAG-TIP60 was a gift from S. H. Baek in the School of Biological Science at Seoul National University. The hTIP60 full-length coding region was amplified from hTIP60 cDNA in the human brain library (Clontech) using PCR and was introduced to pCRII-TOPO vector (Invitrogen), which was called pCRII-TOPO-TIP60. The hTIP60 clone was verified by DNA sequencing. TIP60 full-length (FL) was amplified from pCRII-TOPO-TIP60 using the PCR reaction: forward, 5′-GAA TTC ATG GCG GAG GTG GGG; reverse, 5′-TCT AGA TCA CCA CTT CCC CCT. TIP60-chromo, -Zn, and -HAT truncated mutants were amplified from TIP60-FL using PCR reaction; for TIP60-chromo, forward 5′-GAA TTC ATG GCG GAG GTG GGG-3′, reverse 5′-TCT AGA GAG GAC ATG TGT-3′; for TIP60-Zn, forward, 5′-GAA TTC ACC CCC ACT AAG AAC-3′, reverse, 5′-TCT AGA ATT GTA GTC TTC CGT-3′; for TIP60-HAT, forward, 5′-GAA TTC GTG GCC TGC ATC CTA-3′, reverse, 5′-TCT AGA TCA CCA CTT CCC CCT-3′. These PCR products were introduced into the pCRII-TOPO vector (Invitrogen) and subcloned into the pCDNA-HA vector between EcoRI and XbaI and then verified by DNA sequencing. pCDNA-MDM2 and pCDNA3-HA-p73β have been described previously (28, 29).

Apoptosis Assays and Fluorescence-activated Cell Sorting Analysis—To analyze cellular apoptosis, HEK293 and HeLa cells were grown to 60–70% confluence in complete media then transfected with plasmids encoding p73β in the presence or absence of a TIP60 expression plasmid. Apoptotic cells were identified by their rounded morphology compared with the spread-out morphology of non-apoptotic cells. The number of apoptotic cells was counted and presented as a percentage *versus* the total population of green fluorescence protein (GFP)-empty expressing cells. Chromatin condensation as an apoptotic marker was visualized by Hoechst 33258 (Sigma-Aldrich) staining. H1299 cells were transiently cotransfected with the expression plasmids for HA-p73β, pCDNA-MDM2, and FLAG-TIP60 with or without siRNA of p73 and shRNA of TIP60. Control transfection was performed with the empty plasmid. Forty-eight hours after transfection, cells were stained with trypan blue, and apoptotic cells in at least three different fields (more than 300 cells in one field) was measured. The apoptotic cells were additionally measured by fluorescence-activated cell sorting analysis. Cells were trypsinized, washed with phosphate-buffered saline (PBS), and fixed in 70% ethanol. Fixed cells were re-washed with PBS and stained with 100 μg/ml propidium iodide and 5 μl of annexin V-fluorescein isothiocyanate (51-65874X) purchased from Sigma-Aldrich and BD Biosciences, respectively. Stained cells were analyzed on a FACScan flow cytometer (BD Biosciences) for counting of apoptosis-positive cells.

Luciferase Assay—HEK293, HeLa, and NIH3T3 cells were cultured in 60-mm dishes and transfected with the firefly luciferase p21 reporter gene (0.1 μg) and pCMV-β-galactosidase (0.1 μg) together with pcDNA-HA-p73β, pcDNA3-MDM2, and/or pEGFP-TIP60 using Lipofectamine 2000. After 24 h of transfection, cells were lysed in reporter lysis buffer (Promega). Cell extracts were analyzed with the luciferase reporter assay system using a Lumat LB 9501 Berthold Luminometer. Luciferase activities of the p21-luciferase vector were normalized based on β-galactosidase activity of the cotransfected vector.

In Vivo Binding Assay and Western Blotting—HEK293, HeLa, and SK-OV3 cells were seeded in 100-mm plates at an initial density of 2×10^6 cells and allowed to grow for 12 h. The cells were transfected with the respective plasmids, further incubated for 24 h, and lysed in a buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% SDS, 1% Nonidet P-40, and 1 mM phenylmethanesulfonyl fluoride. The cell suspensions were incubated on ice for 20 min and centrifuged at 12,000 rpm at 4 °C for 20 min. For immunoprecipitation assays the supernatants were pre-cleaned with 20 μl of protein A/G-agarose bead (50% slurry) and then incubated at 4 °C overnight with 30 μl of fresh protein A/G bead in the presence of appropriate antibodies. The beads were washed three times in PBS, resuspended in SDS sample buffer, and boiled for 10 min. Samples were analyzed by Western blotting using the appropriate antibodies to detect protein expression. The polyclonal antibody against the p73 protein generated from glutathione *S*-transferase-p73β was raised in the rabbit (29). Polyclonal antibodies against TIP60 (sc-5725), MDM2 (sc-965), β-tubulin (sc-397), Bax (sc-493), and HA (sc-805) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal antibodies against GFP (GFP-1814 460) and FLAG-M2 were purchased from Roche Diagnostics and Sigma-Aldrich, respectively.

Immunofluorescence Staining and Confocal Microscopic Detection—HeLa cells were grown on a sterile coverslip in 60-mm dishes and transfected with indicated expression vectors using Lipofectamine 2000. Twenty-four hours after transfection cells were fixed with 4% formaldehyde and incubated

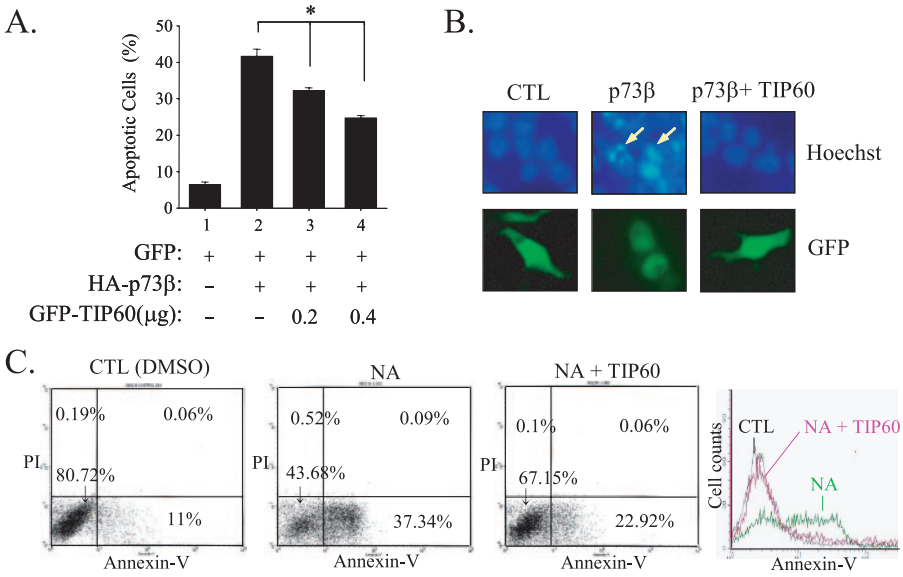

FIGURE 1. **TIP60 inhibits p73β-mediated apoptosis.** *A*, HEK293 cells were transiently co-transfected with CMV-HA-p73β and pEGFP-TIP60. Apoptotic cell percentage induced by p73β was measured in the presence or absence of TIP60. *B*, chromatin condensed apoptotic cells (*arrows*) were visualized by Hoechst 33258 staining in control (*CTL*), p73β-transfected, and p73β plus TIP60-co-transfected HEK cells. *C*, apoptosis was analyzed by flow cytometry using FLAG-TIP60 transfected HeLa cells, which were treated with or without 20 μM NA for 36 h. All data are representative of three independent experiments, and statistical significance is represented by Tukey's post hoc test (*, $p < 0.05$).

with mouse anti-MDM2 antibody (1:500) (Santa Cruz Biotechnology) and rabbit anti-HA antibody (1:500) (Santa Cruz Biotechnology) followed by Cy3-conjugated goat anti-mouse and Cy5-conjugated goat anti-rabbit antibodies (Amersham Biosciences). Plates were washed three times in PBS, and confocal imaging was performed with a Carl Zeiss LSM-510META laser scanning microscope (Oberkochen, Germany).

Preparation of Subcellular Fractions—HeLa cells were washed with ice-cold PBS, harvested by centrifugation, and lysed in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride) for 15 min. For cell lysis 10% (v/v) of Nonidet P-40 was added, and the cells were vortexed for 10 s. After centrifugation at 5000 rpm for 30 s, the supernatant (cytosolic extracts) was transferred to a new tube. The pellet was added to ice-cold buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride), incubated for 15 min at 4 °C, and centrifuged at 14,000 rpm for 5 min. The supernatant (nuclear extracts) was transferred to new tubes and kept frozen at −70 °C until use.

RNA Interference—To knock down endogenous p73 and TIP60, siRNA directed against human p73 (sc-36167, Santa Cruz) and shRNA for hTIP60 were introduced into H1299 cells using Lipofectamine 2000 following the manufacturer's instructions. Forty-eight hours after transfection whole cell lysates were prepared and analyzed for the expression levels of p73 and TIP60 by immunoblotting.

Chromatin Immunoprecipitation (ChIP)—The ChIP assay was performed following a protocol provided by Upstate Biotechnology, Inc. (Lake Placid, NY). In brief, H1299 cells were treated with 20 μM NA. Twenty-four hours after NA treatment cells were cross-linked with 1% formaldehyde in medium for 15 min at 37 °C. Cells were then washed with ice-cold PBS and resuspended in 200 μl of SDS-sample buffer containing a protease inhibitor mixture. The suspension was sonicated 3 times for 10 s with a 1-min cooling period on ice and precleared with 20 μl of protein A-agarose beads blocked with sonicated salmon sperm DNA for 30 min at 4 °C. The beads were removed, and the chromatin solution of each experimental group was immunoprecipitated overnight with anti-p53, -p73, -MDM2, and -TIP60 antibodies at 4 °C followed by incubation with 40 μl of protein A-agarose beads for an additional 1 h at 4 °C. The immune complexes were eluted with 100 μl of elution buffer (1% SDS and 0.1 M $NaHCO_3$), and formaldehyde cross-links were reversed by heating at 65 °C for 6 h. Proteinase K was added to the reaction mixtures and incubated at 45 °C for 1 h. DNA of the immunoprecipitates and control input DNA was purified using the phenol/chloroform extraction method and then analyzed by regular PCR using the human p21 promoter-specific primers. The primer sequences were 5′-CAC CTT TCA CCA TTC CCC TA-3′ and 5′-GCA GCC CAA GGA CAA AAT AG-3′ for p21.

Statistical Analysis—Statistical analysis of variances between two different experimental groups was performed with Tukey's post hoc comparison test using SPSS (Version 11.5). All experiments were repeated at least three times. The levels are considered significant for $p < 0.05$ (shown as a single *asterisk*), very significant for $p < 0.01$ (shown as double *asterisks*), obviously significant for $p < 0.001$ (shown as triple *asterisks*), and not significant (*n.s.*).

RESULTS

p73β-induced Apoptotic Cell Death Is Inhibited by TIP60—TIP60 was controversially reported to be a transcriptional co-activator (20, 22) and a co-repressor for certain transcriptional regulators (23, 30). Thus, we determined apoptotic patterns of HEK293 cells after transient co-transfection with p73β and TIP60 expression plasmids to investigate how TIP60 regulates p73β-mediated apoptosis. Apoptotic rate was assessed by counting the morphologically round and shrunken p73β-transfected cells in the absence or presence of TIP60. As indicated in Fig. 1*A*, p73β-induced apoptosis was significantly blocked by TIP60 in a dose-dependent manner. The chromatin-condensed pattern of p73β-induced apoptotic cells, which was visualized by Hoechst 33258 staining, was markedly decreased in the presence of TIP60 (Fig. 1*B*). Because endogenous p73β induced by a naphthoquinone analog (NA) was reported to augment cellular apoptotic responses (29), we next determined the effects of TIP60 on endogenous p73β-induced apoptosis. After TIP60-transfected HeLa cells and their controls were treated with 20

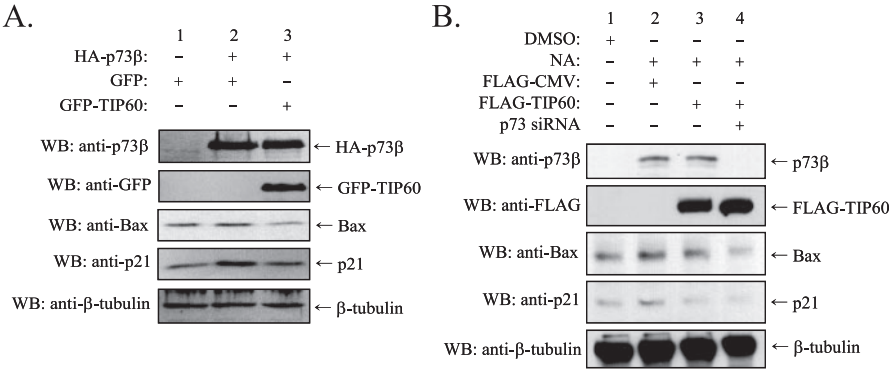

FIGURE 2. **Negative regulation of p21 and Bax expression by TIP60.** *A*, HEK293 cells were transiently co-transfected with expression plasmids of p73β and TIP60. Whole cell extracts were prepared and subjected to Western blotting using anti-p21 and anti-Bax antibodies. pEGFP-empty vector served as a negative control for TIP60 expression. β-Tubulin was used as loading control. *B*, H1299 cells were transiently transfected with FLAG-CMV-empty, pFLAG-CMV-TIP60, and p73 siRNA, and then cells were treated with 20 μM NA for 48 h. Whole cell lysates were immunoblotted with the indicated antibodies.

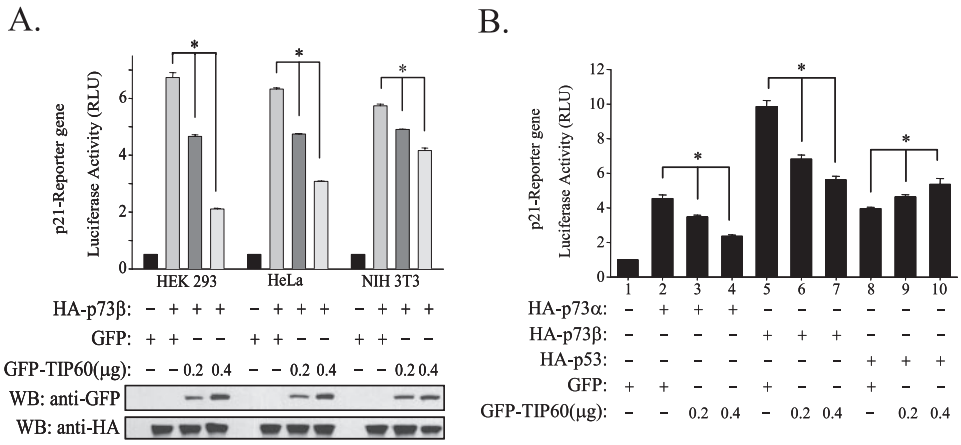

FIGURE 3. **TIP60 represses transcriptional activity of p73β.** *A*, HEK293, HeLa, and NIH3T3 cells were transiently co-transfected with HA-p73β, increased amounts of GFP-TIP60 (0.2 and 0.4 μg), and a luciferase-reporter plasmid containing the p21 promoter and pCMV-β-galactosidase. Luciferase activity was measured 36 h after transfection. Whole cell lysates were immunoblotted with anti-GFP and anti-HA antibodies. *B*, TIP60 inhibits transactivity of p73 but not p53. HEK293 cells were transiently co-transfected with HA-p73α, HA-p73β, and HA-p53 with increasing amounts of the TIP60 expression plasmid (0.2, 0.4 μg). Luciferase activity was measured 36 h after transfection. All data were normalized to β-galactosidase activity. The data are expressed in relative fold increase of luciferase units (*RLU*). All data are representative of three independent experiments, and statistical significance is represented by Tukey's post hoc test (*, $p < 0.05$).

μM NA, their apoptotic patterns were analyzed by flow cytometry (Fig. 1*C*). The annexin-V-stained portion, which represents an apoptotic pattern, was significantly increased to 37.4% in NA-treated HeLa cells compared with 11% in control cells. In contrast, TIP60-transfected cells showed 22.92% of the apoptotic ratio at the same experimental condition (Fig. 1*C*). Taken together, these results indicate that TIP60 represses increased apoptosis, which is induced by exogenous and endogenous p73β.

TIP60 Reduces the Expression Level of Apoptotic Signaling Proteins Such as p21 and Bax, Which Are Induced by p73β—Because p73β induces cellular apoptosis by activating its downstream target proteins such as p21 and Bax (31), we next examined the expression level of p21 and Bax by Western blot analysis using HEK293 cells co-transfected with HA-p73β and GFP-TIP60 expression plasmids. As indicated in Fig. 2*A*, the enhanced expression level of p21 and Bax by p73β overexpression was significantly reduced by TIP60. We next examined the effects of TIP60 on the expression of p21 and Bax, which was enhanced by NA-induced endogenous p73β in p53 null H1299 cells. TIP60 also significantly reduced the expression level of p21 and Bax enhanced by endogenous p73β in H1299 cells (Fig. 2*B*, *lane 3*) and HeLa cells (supplemental Figs. S1, *lane 3*). In addition, p73 siRNA did markedly inhibit Bax and p21 expression induced by NA treatment (Fig. 2*B*, *lane 4*), suggesting that TIP60 represses Bax and p21 expression, which are specifically modulated through the p73-related pathway. Taken together, these results indicate that TIP60 negatively regulates the expression of p73β downstream target proteins such as p21 and Bax in a p53-independent manner.

p73β Transcriptional Activity Is Repressed by TIP60—Because TIP60 reduces p73β-mediated apoptosis by inhibiting p73-downstream target genes, we investigated the effects of TIP60 on p73β transcriptional activity using HEK293, HeLa, and NIH3T3 cell lines, which were transiently co-transfected with a p73β expression plasmid plus a luciferase reporter plasmid containing the p73-responsive element from the p21 promoter. As indicated in Fig. 3*A*, enhanced luciferase activities of the three cell lines by p73β transactivation were significantly repressed by TIP60 in a dose-dependent manner. These results demonstrate that p73β-mediated transactivation is specifically inhibited by TIP60 in various cell lines independent of p53 expression.

TIP60 Represses Transactivation of p73 but Not of p53—The p73 gene encodes at least two different proteins, termed p73α and p73β, that differ exclusively at their C-terminal portions due to an alternative splicing event (32). To examine whether TIP60 can regulate transactivation of p53 and p73 splice variants such as p73α and p73β, we co-transfected HEK293 cells with combined expression plasmids of HA-p73α, HA-p73β, and HA-p53 along with a GFP-tagged TIP60 expression vector as indicated in Fig. 3*B*. In the presence of TIP60, transcriptional activities of both p73 variants were significantly inhibited in a dose-dependent manner; however, p53 transactivation was increased with TIP60 expression compared with its control (Fig. 3*B*). Taken together, these results demonstrate that TIP60 can repress transcriptional activity of both p73 splicing variants, p73α and p73β, but not that of p53.

TIP60 Does Not Recruit Histone Deacetylase (HDAC) to Repress p73β Transactivation—Recent studies have suggested that TIP60 acts either as a co-activator or a co-repressor to modulate transcriptional activities by associating with various transcriptional regulators. For example, TIP60 has been implicated in the negative regulation of interleukin-9-induced c-*myc* expression through STAT3 repression, which was associated with the recruitment of HDAC7 by TIP60 (30). Thus, we tested whether TIP60 represses p73β transactivation through cooperation with HDACs. First, we incubated HEK293 cells co-transfected with p73β and TIP60 in culture media containing trichostatin A (an inhibitor of class I and II histone deacetylases) for 12 h. As indicated in Fig. 4, TIP60 repression of enhanced luciferase activities by p73β transactivation was not recovered by 1 or 5 μM trichostatin A treatment. These results indicate that TIP60 does not require HDAC activity to repress p73β transactivation.

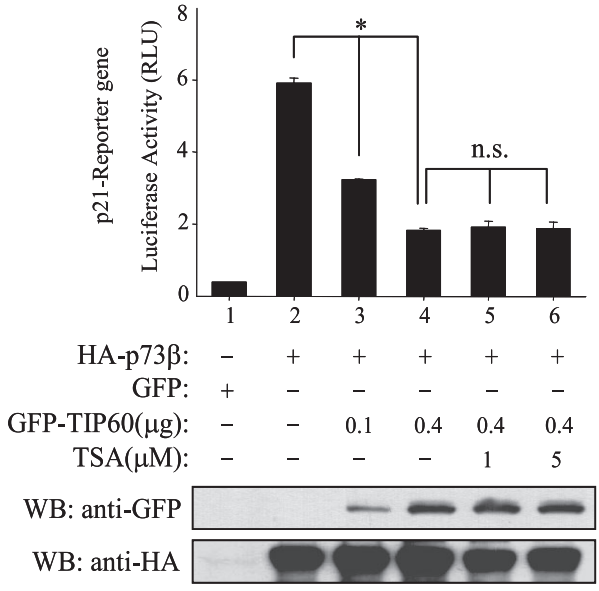

FIGURE 4. **TIP60 does not recruit HDAC to reduce transcriptional activity of p73β.** HEK293 cells were transiently co-transfected with HA-p73β and indicated amounts of GFP-TIP60 (0.1, 0.4 μg) in the presence or absence of trichostatin A (*TSA*; 1 and 5 μM) for 12 h. p73β transcriptional activity was measured by RLU. The protein expression levels in various experimental conditions were assessed by immunoblotting using anti-GFP and anti-HA antibodies. All data are representative of three independent experiments, and statistical significance is represented by Tukey's post hoc test (*, $p < 0.05$; *n.s.*, not significant).

Interaction between p73β and TIP60 Is Mediated by MDM2—It has recently been reported that TIP60 interferes with MDM2-mediated degradation of p53 to maintain a basal pool of p53 under normal growth conditions (25). MDM2 was also reported to physically interact with TIP60 and induce its ubiquitination and proteasome-dependent degradation (33). Therefore, we investigated possible MDM2 regulation in TIP60 repression of p73β transactivation. After HEK293 cells were co-transfected with HA-tagged p73β and GFP-fused TIP60 expression plasmids along with an MDM2 expression vector, the whole cell lysates were immunoprecipitated with anti-GFP antibodies. The immunoprecipitated proteins were then analyzed by Western blotting using specific anti-HA and anti-GFP antibodies to determine p73β and TIP60, respectively (Fig. 5*A*). In the presence of MDM2, the amount of p73β proteins co-immunoprecipitated with TIP60 (Fig. 5*A*, *lane 3*) was significantly increased compared with that in the absence of MDM2 (Fig. 5*A*, *lane 2*). HEK293 cells transfected with p73β alone served as a negative control (Fig. 5*A*, *lane 1*). Likewise, p53-null SK-OV3 co-transfected with HA-tagged p73β and FLAG-tagged TIP60 expression plasmids in the presence of MDM2 showed the pattern of co-immunoprecipitated p73β and TIP60 (Fig. 5*B*, *lane 2*) in comparison to the absence of MDM2 (Fig. 5*B*, *lane 1*). The total amounts of p73β and TIP60 in whole cell lysates were almost equal in both experimental conditions (bottom in Fig. 5, *A* and *B*). Taken together, these results indicate that the interaction of TIP60 with p73β is physically mediated by MDM2.

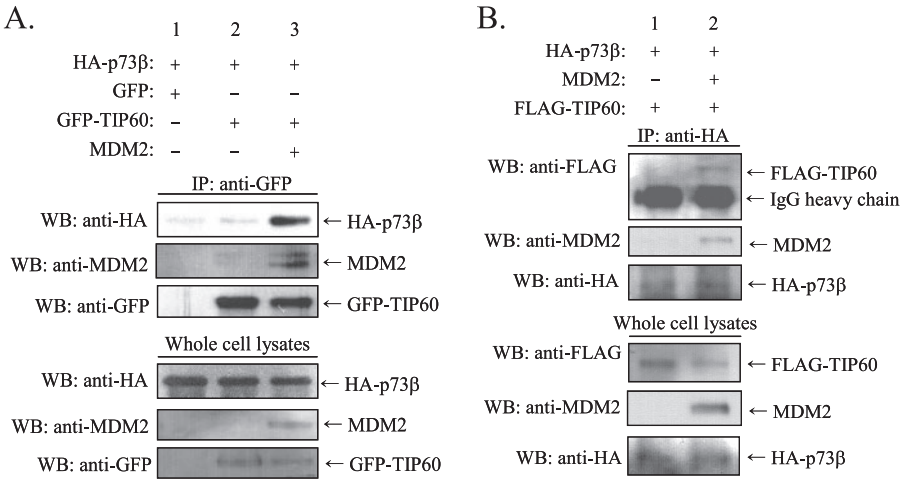

FIGURE 5. **The interaction of TIP60 with p73β is mediated by MDM2.** *A*, HEK293 cells were transfected with expression plasmids of HA-tagged p73β together with either pEGFP (*lane 1*), pEGFP-TIP60 (*lane 2*), or pEGFP-TIP60 plus pCDNA-MDM2 (*lane 3*). After whole cell lysates were immunoprecipitated (*IP*) with anti-GFP antibody, a Western blot was performed using indicated antibodies. *B*, SK-OV3 cells were transfected with FLAG-tagged TIP60 together with HA-p73β (*lane 1*) or HA-p73β plus pCDNA-MDM2 (*lane 2*). Whole cell lysates were immunoprecipitated with anti-HA antibody, and Western blot analysis was performed using indicated antibodies.

MDM2 Is a Bridge of Complex Formation between TIP60 and p73β—To further confirm the MDM2-mediated physical interaction between p73β and TIP60, we investigated the effect of MDM2 immunodepletion in HeLa cells co-transfected with TIP60 and p73β expression plasmids with or without the MDM2 expression plasmid. When MDM2 proteins were immunodepleted with 0.2–0.8 μg of anti-MDM2 monoclonal antibodies using protein agarose A/G beads from HeLa whole cell lysates, the level of MDM2 proteins was determined by Western blot analysis with anti-MDM2 antibodies. As indicated in Fig. 6, *Supernatant* (*second*) *panel* MDM2 proteins in the supernatant were significantly reduced by 0.8 μg of anti-MDM2. Next, we

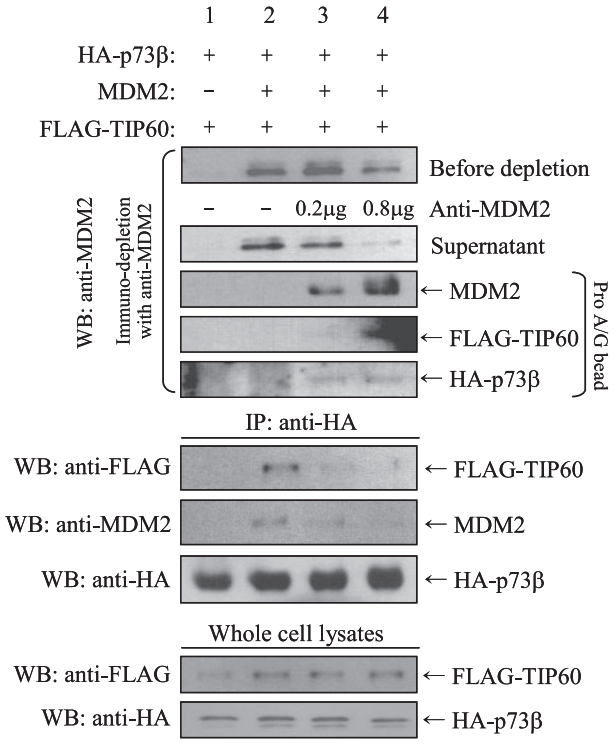

FIGURE 6. **MDM2-mediated complex formation between TIP60 and p73β.** HeLa cells were co-transfected with HA-p73β and FLAG-TIP60 expression plasmids together with or without the MDM2 expression plasmid. Whole cell lysates were incubated with increased amounts of anti-MDM2 antibodies (0.2 and 0.8 μg) in the presence of protein A/G-agarose beads for MDM2 immunodepletion. After the supernatants were immunoprecipitated (*IP*) with anti-HA antibodies, Western blot analysis was performed with anti-FLAG, anti-HA, and anti-MDM2 antibodies. Bead-bound proteins also were detected by Western blotting using anti-FLAG and anti-HA antibodies. Whole cell lysates were analyzed by Western blotting using anti-FLAG and anti-HA antibodies for the control.

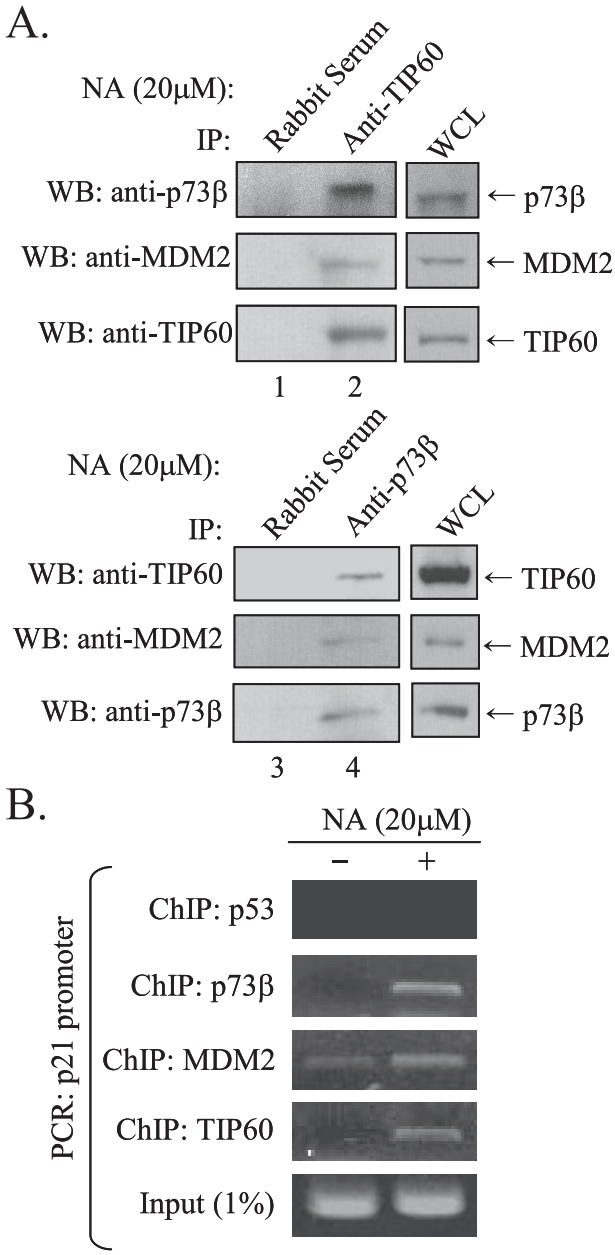

FIGURE 7. **TIP60·MDM2·p73β ternary complex formation and its association with the p21 promoter *in vivo*.** *A*, after HeLa cells were stimulated with 20 μM NA for 12 h, the *in vivo* interaction between endogenous TIP60 and p73β was determined by immunoprecipitation (*IP*) using anti-TIP60 (*lanes 1* and *2*) and anti-p73 (*lanes 3* and *4*) antibodies and by Western blot analysis using anti-p73, anti-MDM2, and anti-TIP60 antibodies. Each protein was normalized to its expression in whole cell lysates (*WCL*). *B*, H1299 cells were grown with or without 20 μM NA; induction was for 12 h, and ChIP assays were performed with p53, p73β, MDM2, and TIP60 antibodies. Then PCR was performed using a primer set that flanked the 224 bp region of p21 promoter.

immunoprecipitated HA-tagged p73β in the supernatants with anti-HA antibodies and performed Western blot analysis using anti-FLAG, anti-HA, and anti-MDM2 antibodies (Fig. 6, *middle panels*. The amount of co-immunoprecipitated FLAG-TIP60 with HA-p73β was significantly decreased in a MDM2 depletion-dependent manner compared with almost the same level of immunoprecipitated p73β (Fig. 6, *middle panels*. Concomitant with immunodepletion of MDM2, co-immunoprecipitated TIP60 and p73β are increased in a protein A/G bead-bound MDM2 complex (Fig. 6, *Prot A/G bead*). Almost the same level of expressed FLAG-TIP60 and HA-p73 in the whole cell lysates served as the control in this study (Fig. 6, *lower panels*). Taken together, these results indicate that the interaction of TIP60 with p73β is bridged by MDM2 as a ternary complex.

TIP60·MDM2·p73β Ternary Complex Is Localized in the Nucleus—To determine the localization of the TIP60·MDM2·p73β ternary complex in the nucleus, we immunostained HeLa cells, which were co-transfected with GFP-fused TIP60, MDM2, and HA-tagged p73β expression plasmids using anti-GFP, anti-MDM2, and anti-HA antibodies. As shown in supplemental Fig. 2*A*, p73β (*blue*), MDM2 (*red*), and TIP60 (*green*) were co-localized in the nucleus under confocal microscopy. For further confirmation of the TIP60·MDM2·p73β ternary complex in the nucleus, we examined the nuclear co-localization and the interaction of the TIP60·MDM2·p73β ternary complex by Western blot analysis. After HeLa cells were co-transfected with HA-tagged p73β, FLAG-tagged TIP60, and MDM2 expression plasmids, both nuclear and cytosolic fractions were separated and verified by Western blot analysis using antibodies for each marker protein, which were Rb for nucleus and β-tubulin for cytoplasm (supplementalFig. S2*B*). The nuclear extracts were then immunoprecipitated with anti-HA antibodies, and Western blot

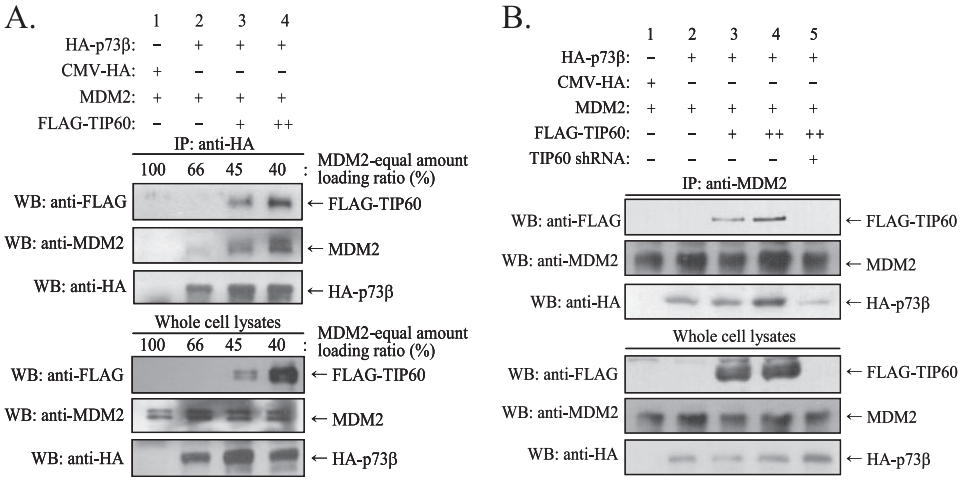

FIGURE 8. **The binding affinity of MDM2 to p73β is enhanced by TIP60.** *A*, HeLa cells were transiently transfected with HA-tagged p73β, MDM2, and FLAG-tagged TIP60 expression plasmids together with or without the CMV-HA control vector. MDM2-equal amount loading ratio (%) was calculated based on each MDM2 expression ratio, which was determined by an Image analyzer (Bio-Rad) using Quantity One software. After the corresponded amounts of whole cell lysates were immunoprecipitated (*IP*) with anti-HA antibodies, Western blot analysis was then performed with anti-MDM2, anti-HA, and anti-FLAG antibodies. The data are representative of three independent experiments. *B*, HEK293 cells were transiently transfected with HA-tagged p73β, MDM2, and FLAG-tagged TIP60 expression plasmids together with or without the TIP60 shRNA. After the corresponded amounts of whole cell lysates were immunoprecipitated (*IP*) with anti-MDM2 antibodies, Western blot analysis was then performed with anti-MDM2, anti-HA, and anti-FLAG antibodies. The data are representative of three independent experiments.

analysis was performed using anti-FLAG, anti-HA, and anti-MDM2 antibodies. As indicated in supplemental Fig 2*C*, FLAG-tagged TIP60 was specifically immunoprecipitated with HA-tagged p73β in the presence of MDM2 (supplemental Fig. S2*C*, *lane 2*) compared with in the absence of MDM2 (supplemental Fig. S2*C*, *lane 1*) even though all proteins were markedly detected in the whole cell lysates. Therefore, we demonstrate that TIP60 specifically localizes and interacts with p73β through MDM2 in the nucleus.

TIP60·MDM2·p73β Ternary Complex Associates with p73-target p21 Promoter in Vivo—To evaluate the ternary complex of TIP60·MDM2·p73β *in vivo*, both endogenous TIP60 and p73β in whole cell lysates of cultured HeLa cells, which were induced by 20 μM NA, were immunoprecipitated with anti-TIP60 (Fig. 7*A*, *lanes 1* and *2*) and anti-p73β polyclonal antibodies (Fig. 7*A*, *lanes 3* and *4*) together with their control rabbit serums, respectively. Western blot analysis was then performed with anti-TIP60, anti-p73β, and anti-MDM2 antibodies. We clearly observed that p73β and MDM2 were specifically detected by immunoprecipitation with the anti-TIP60 antibody (Fig. 7*A*, *lane 2*) but not with the preimmune rabbit serum (Fig. 7*A*, *lane 1*). Conversely, endogenous TIP60 and MDM2 were markedly immunoprecipitated with anti-p73β antibody (Fig. 7*A*, *lane 4*) but not with control rabbit serum (Fig. 7*A*, *lane 3*). For the control, an almost even expression level of p73β, TIP60, and MDM2 in whole cell lysates was detected from anti-TIP60 and anti-p73β-immunoprecipitated experimental conditions, respectively (Fig. 7*A*, *WCL*). These results suggest that physical interaction between p73β and TIP60 requests MDM2 to form the ternary complex in a linearized manner *in vivo*.

To determine whether TIP60, MDM2, and p73β are concurrently associated with the p21 promoter in a p53-independent manner, we performed a ChIP experiment using p53 null H1299 cells (Fig. 7*B*). The basal p73β binding to the p21 promoter was increased after 20 μM NA treatment, which is undoubtedly related to increased levels of p73β caused by treatment with this agent. Endogenous TIP60 and MDM2 are also associated with p21 promoters in NA-treated H1299 cells. However, p53 does not associate with p21 promoter by NA treatment. Thus, there was a strong binding correlation between TIP60·MDM2·p73β and the p21 promoter in a p53-independent manner.

TIP60 Enhances Binding Affinity of MDM2 to p73β—To test whether TIP60 can affect p73β binding affinity to MDM2, HeLa cells were transiently transfected with HA-tagged p73β, MDM2, and FLAG-tagged TIP60 expression plasmids (Fig. 8*A*). Based on the MDM2 expression ratio, we calculated the percent of MDM2 equal amount loading ratio of each transfected cell (100:66:45:40 of the same above transfectants) to adjust the same amount of MDM2 for testing its p73β binding affinity (Fig. 8*A*, *bottom panel*). Next, we immunoprecipitated the whole cell lysates with anti-HA antibody, and Western blot analysis was performed with anti-MDM2, anti-HA, and anti-FLAG antibodies. Co-precipitated MDM2 with HA-tagged p73β was significantly increased in a TIP60 dose-dependent manner (*lanes 3* and *4*). To further confirm the binding affinity enhancement of p73β/MDM2 interaction by TIP60, we used TIP60 shRNA transfectants of HEK293 cells and immunoprecipitated with anti-MDM2 antibodies (Fig. 8*B*). Co-precipitated p73β with MDM2 was significantly increased in a TIP60 dose-dependent manner (*lanes 3* and *4*). However, knock-down of TIP60 reduced co-precipitated p73β with MDM2 (*lane 5*). These results indicate that TIP60 markedly enhances MDM2 binding affinity to p73β.

TIP60 Represses p73β Transactivation by Interacting with MDM2—In a previous report p73β binding to MDM2 was shown to result in a dramatic inhibition of p73β transactivity (13). Therefore, we hypothesized that TIP60 may enhance MDM2-mediated inhibition of p73β transactivity through a TIP60·MDM2·p73β ternary complex. To verify the hypothesis, we first investigated the TIP60 binding region of MDM2. Based on TIP60 functional domains, we constructed TIP60 mutants containing its chromo, zinc finger, and HAT domain (Fig. 9*A*). First, HEK293 cells were transfected with combined expression plasmids of TIP60 full-length, chromo, zinc finger, HAT domain together with MDM2. After the whole cell lysates were immunoprecipitated with anti-HA antibodies, Western blot analysis was performed with anti-MDM2 monoclonal antibodies and anti-HA antibodies. As shown in Fig. 9*B* neither the chromo nor HAT domain of TIP60 bound to MDM2; in contrast, TIP60 full-length and zinc finger domain containing

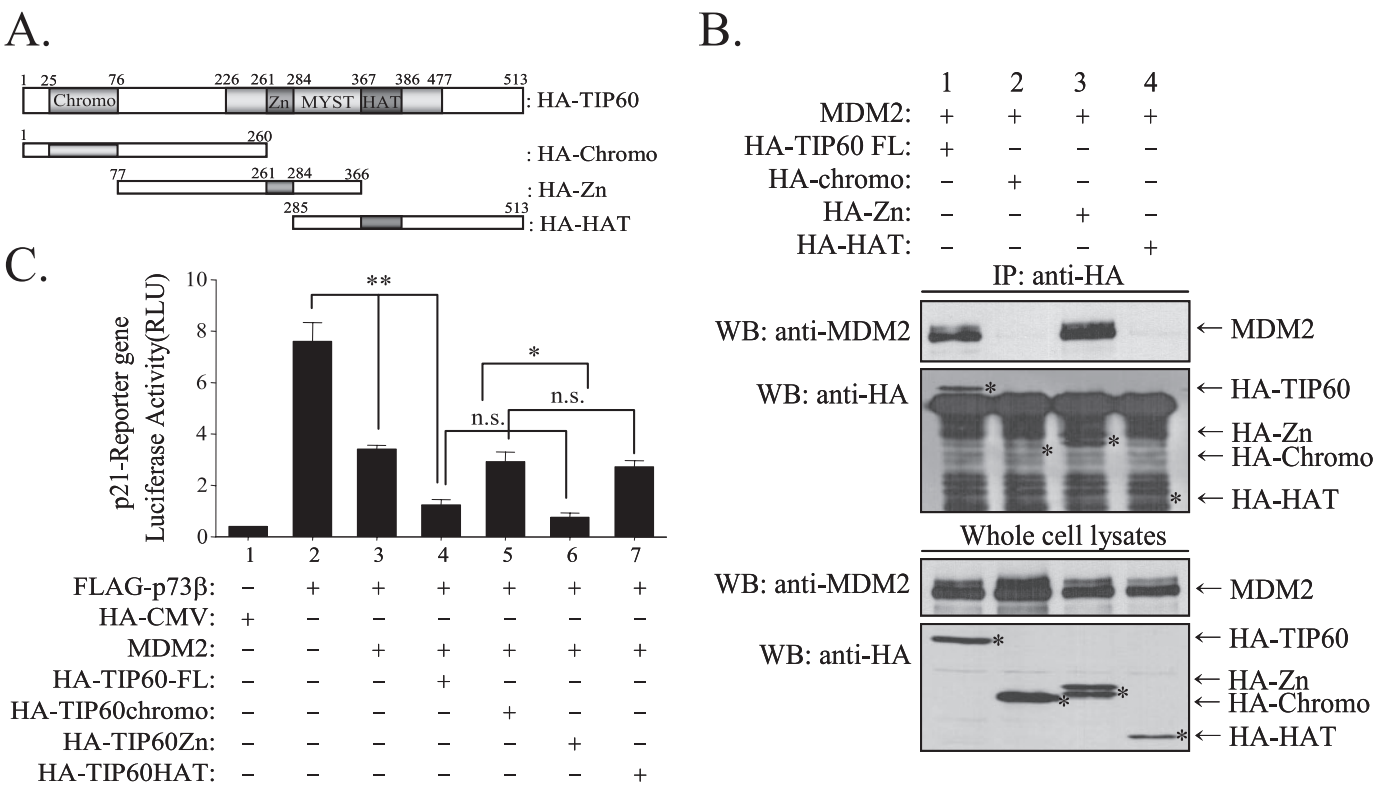

FIGURE 9. **TIP60-mediated repression of p73β transactivation through interacting with MDM2.** *A*, schematic representation of TIP60 protein domains. TIP60 encodes 513 amino acids, which contains an N-terminal chromo domain, zinc finger domain, and a C-terminal conserved MYST domain. The MYST domain contains a short sequence, residues 367–386, which consists of the catalytic subunit conserved HAT (histone acetyltransferase) domain. We constructed three different expression plasmids which contain HA-tagged chromo, zinc finger, and HAT domains. *B*, HEK293 cells were co-transfected with expression plasmids of MDM2 plus three different TIP60 domains in *panel A*. After the whole cell lysates were immunoprecipitated (*IP*) with anti-HA, the expression of MDM2, TIP60, and three TIP60-truncated mutants was determined by Western blot analysis using anti-MDM2 and anti-HA antibodies, respectively (*). As controls, total protein amounts of MDM2, HA-TIP60, and HA-tagged zinc finger, chromo, and HAT domains of TIP60 in the same whole cell lysates were also analyzed by Western blotting using specific anti-MDM2 and anti-HA antibodies, respectively. *C*, HEK293 cells were co-transfected with combined expression plasmids of FLAG-p73β, MDM2, and HA-tagged FL, zinc finger, chromo, and HAT domains of TIP60 together with a luciferase reporter plasmid containing the p21 promoter. Luciferase activities were measured 36 h after the transfection as described under "Experimental Procedures." The data were normalized to β-galactosidase activity and are expressed in relative -fold increase of luciferase units (*RLU*). All data are representative of three independent experiments, and statistical significance is represented by Tukey's post hoc test (*, $p < 0.05$; **, $p < 0.01$; *n.s.*, no significant).

amino acids 261–284 markedly interacted with MDM2. The total expression of MDM2, TIP60 full-length, zinc finger, chromo, and HAT domain of TIP60 in whole cell lysates was detected by Western blot analysis as controls (Fig. 9*B*, *lower panels*). Next, we measured p73β transcriptional activity in the presence of full-length TIP60 or truncated TIP60 to determine the necessity of the interaction of TIP60 with MDM2 for p73β functional repression. Consistent with the previous report, MDM2 overexpression significantly repressed p73β transactivation (Fig. 9*C*, *lane 3*). The repression of p73β transcriptional activation was significantly enhanced by the full-length TIP60 and the zinc finger domain of TIP60 (*lanes 4* and *6*) not by the chromo and HAT domain of TIP60 (*lanes 5* and *7*), indicating that physical association with the zinc finger domain of TIP60 is necessary for enhanced repression of p73β transactivation.

Repression of p73β-induced Apoptosis Is Specifically Accelerated by TIP60 via Interaction with MDM2—We next investigated the role of the interaction of TIP60 with MDM2·p73β in MDM2-mediated repression of p73β-induced apoptosis in H1299 cells. The number of apoptotic cells induced by p73β was significantly decreased by overexpressed MDM2 (Fig. 10*A*, *lane 4*) compared with the control (Fig. 10*A*, *lane 3*). The inhibitory effect of MDM2 was markedly accelerated by overexpression of the HA-tagged full-length TIP60 (Fig. 10*A*, *lane 5*) and zinc finger domain (*lane 7*), which interact with MDM2. However, p73β-mediated apoptosis was not repressed by the TIP60-chromo (*lane 6*) and HAT (*lane 8*) domain, which does not bind to MDM2. Knockdown of TIP60 using its shRNA specifically eliminated MDM2-mediated reduction of p73β-induced apoptosis (Fig. 10*B*, *lanes 5* and 6; supplemental Fig. S3, *lane 6*). To investigate that inhibited apoptotic responses by the ternary complex are specifically modulated though the p73 pathway, we used p73-siRNA transfectants of p53 null H1299 cells, in which the expression of p73 proteins was almost completely abrogated. The ratio of apoptotic cells were significantly reduced after p73-siRNA transfection (Fig. 10*B*, *lane 7*). Therefore, these results demonstrate that TIP60 alleviates p73β-induced apoptotic responses through its interaction with MDM2·p73β in a p53-independent manner.

DISCUSSION

In this study we report that TIP60 forms a ternary complex with p73β, which is bridged by MDM2. We also observe that the TIP60·MDM2·p73 complex negatively regulates p73β-dependent transcriptional activity of a reporter containing the p21 promoter. Furthermore, we demonstrate that TIP60 inter-

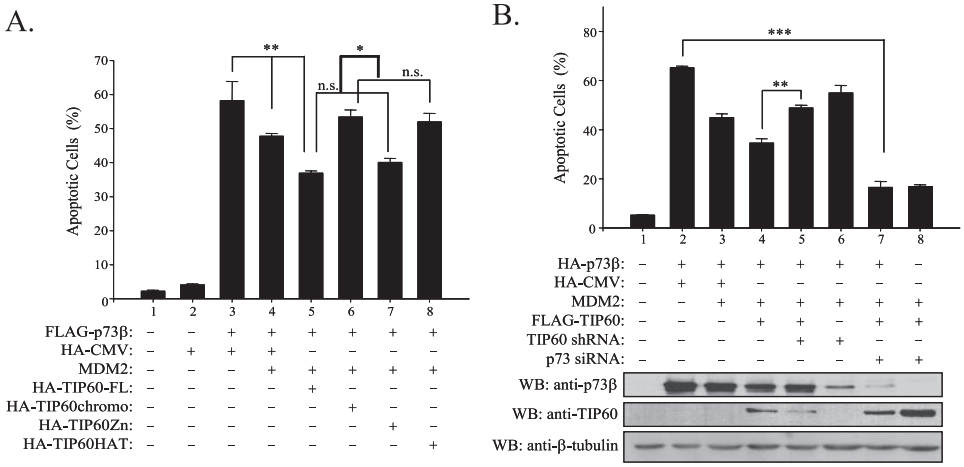

FIGURE 10. **Accelerated repression of p73β-induced apoptosis by TIP60 via interaction with MDM2.** *A*, H1299 cells were co-transfected with FLAG-tagged p73β and MDM2 expression plasmids together with HA-tagged full-length TIP60 (*HA-TIP60-FL*), HA-tagged TIP60-chromo (*HA-TIP60chromo*), zinc finger (*HA-TIP60-Zn*), and HAT domain. Apoptotic cells were determined by the percentage of trypan blue positive cells 48 h after transfection as described under "Experimental Procedures." Transfection with the HA-CMV-empty plasmid alone served as negative control. *B*, H1299 cells were co-transfected with HA-tagged p73β and MDM2 expression plasmids together with FLAG-tagged full-length TIP60 and TIP60 shRNA or p73 siRNA. Apoptotic cells were counted by the percentage of trypan blue-stained cells 48 h after transfection as described under "Experimental Procedures." Endogenous TIP60 and p73 levels were detected by Western blotting using TIP60 and p73 antibodies. β-Tubulin served as a loading control. All data are representative of three independent experiments, and statistical significance is represented by Tukey's post hoc test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *n.s.*, no significant).

acts with MDM2, a repressor of p73, which may result in the enhancement of MDM2 binding to p73β. Consequently, TIP60 increases MDM2-mediated transcriptional repression of p73β and leads to the inhibition of p73β-mediated apoptosis.

It was previously shown that TIP60 possesses intrinsic HAT activity on histone subunits H2A, H3, and H4 *in vitro* using purified histones as substrates (34–36), which suggests potential roles in chromatin remodeling and gene regulation. Except for HAT activity, TIP60 may promote acetylation of cellular proteins other than histones to regulate gene expression. For example, TIP60 enhances the activity of androgen receptor (20, 37) and p53 (26, 27), which demonstrates that TIP60 acts as a co-activator by promoting the acetylation of target proteins but not histones.

Recently, novel functions of TIP60 were reported independently of the TIP60 intrinsic HAT activity. First, TIP60 acts as a co-activator of p53 by interfering with MDM2-mediated p53 degradation by forming a ternary TIP60·MDM2·p53 complex (25). The complex formation leads to increased stability of p53 and enhances p53 target gene expression. Second, TIP60 acts as a co-regulator to repress transcriptional activation. For example, the CREB, a transcriptional activator stimulated by hormone and growth factor-dependent phosphorylation by protein kinase A, is co-repressed by direct binding between TIP60 and CREB (23). TIP60 also negatively regulates the transcription factors such as STAT3 (30), orphan nuclear receptor Reverbβ (38), and Kruppel-like factor 4 (39) through physical interaction with HDACs. Because TIP60 possesses dual roles of co-activator and co-repressor, these reports support the notion that TIP60 and other MYST members may repress gene expression and provide a possible mechanism for the repressive effect of TIP60 on p73-mediated transcriptional activation.

In this aspect the following are possible speculations that may lead to the inhibitory effect of TIP60 on transcriptional activity of p73β; 1) TIP60 acts as a co-repressor by recruiting HDACs to p73 target promoter like the STAT3 and Kruppel-like factor 4 cases; 2) TIP60 recruits another negative regulator of p73 and modulates binding affinity between them. To explain the above possibilities, we tested whether TIP60 recruits HDACs upon p73 transcriptional activity. However, HDAC inhibitor treatment does not recover transcriptional activity of p73β (Fig. 4). These results suggest that TIP60 does not seem to recruit HDACs to inhibit the transcriptional activity of p73β. Interestingly, because we observe that TIP60 physically interacts with p73β through MDM2, which is repressor of p73, p73 negative regulator recruitment was tested. Our study shows that the interaction between TIP60 and p73β needs MDM2 to form a ternary complex in the nucleus (Fig. 5 and supplemental Fig. 2). We also performed an immunodepletion assay to demonstrate MDM2-bridged complex formation. As shown in Fig. 6, immunodepletion of MDM2 protein markedly reduces ternary complex formation among p73β, MDM2, and TIP60. Concomitant with these results, immunoprecipitated MDM2 brings co-precipitation of both TIP60 and p73β; this is strong biochemical evidence of MDM2-bridged ternary complex formation.

MDM2 is one of the major negative regulators of p53 stability via the ubiquitin pathway. MDM2 inhibits the transcriptional activity of p73 as well as p53. Interestingly, p53 is rapidly degraded via the proteasomal pathway in the presence of MDM2; in contrast, p73 is stabilized by MDM2 rather than being degraded (40). In the context of our study, there are several reasons to consider that may explain why increased MDM2-mediated interaction with p73 is associated with decreased transactivation function of p73. The direct binding of MDM2 to the N-terminal transactivation domain of p73 and/or competitive disruption of p300/CBP binding to the N-terminal transactivation domain by MDM2 may inhibit p73 transcriptional activity (13). Furthermore, it is possible that MDM2 regulates NEDD8 modification of lysyl residues (Lys-321, Lys-327, and Lys-331), which are targets of acetylation by the transcriptional co-activators p300/CBP (41). Thus, transcriptional activity of p73 is reduced by post-translational modification of p73 through MDM2. Also, to inhibit transcriptional activity of p73, it is possible that MDM2 recruits corepressors to negatively regulate p73-dependent transcription. Mirnezami *et al.* (42) showed that HDM2, a human homolog of MDM2, could repress the transcriptional activity of p53 through the recruitment of a known transcriptional co-repressor, hCtBP2. It is, therefore, tempting to speculate that MDM2-mediated recruitment of TIP60 as a co-repressor is also an important mecha-

nism to modulate the transcriptional activity of p73, as in the p53 case, through protein-protein interactions.

Furthermore, we tested the physiological significance of binding between TIP60 and p73β through MDM2 by assessing modulations in the transcriptional activity of p73β. TIP60 significantly enhances the repressive effect of MDM2 on p73β-mediated transactivity through binding with MDM2 (Fig. 9). To describe the role of TIP60 role in the MDM2-p73β interaction, we tested the binding affinity between MDM2 and p73β, which was modulated by TIP60. We used equal amount of MDM2 and p73β while increasing the quantity of TIP60. Because ectopically expressed TIP60 enhances the MDM2 level, we balanced the quantity of MDM2 to identify the TIP60 binding effects on an MDM2-p73β-forming ternary complex. Increasing amounts of TIP60 stabilize MDM2-p73β binding more efficiently in a concentration-dependent manner (Fig. 8).

Our results also suggest the intriguing possibility that a ternary TIP60·MDM2·p73 complex could also be involved in the transcriptional regulation of p73β *in vivo*, not in an overexpressed system. Indeed, because MDM2 has been shown by chromatin immunoprecipitation to bind p73-responsive promoters in the presence of p73, it could be envisioned that TIP60, and thereby its co-regulator activity, is recruited to p73-responsive promoters. In our ChIP assay p73β intensively associated with the p21 promoter after NA treatment. However, MDM2 and TIP60 were also recruited to the p21 promoter region by NA. This demonstrates that the ternary complex we found here is bound to the p21 promoter and down-regulates its transcription *in vivo* (Fig. 7*B*).

Despite intensive recent studies, the effects of TIP60 on cell physiology remain to be fully elucidated. In this respect it is important to note that our findings contribute to a functional linkage between TIP60 and p73β in the biological network, showing that TIP60 associates with and regulates p73β by involving its negative regulator, the MDM2 protein, to form the ternary complex TIP60·MDM2·p73β.

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TIP60 Represses Transcriptional Activity of p73β via an MDM2-bridged Ternary Complex

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