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An Essential Role of Human Ada3 in p53 Acetylation*

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The p53 tumor suppressor protein functions as a critical component of genotoxic stress response by regulating the expression of effector gene products that control the fate of a cell following DNA damage. Unstressed cells maintain p53 at low levels through regulated degradation, and p53 levels and activity are rapidly elevated upon genotoxic stress. Biochemical mechanisms that control the levels and activity of p53 are therefore of great interest. We and others have recently identified hAda3 (human homologue of yeast alteration/deficiency in activation 3) as a p53-interacting protein and enhancer of p53 activity. Here, we show that endogenous levels of p53 and Ada3 interact with each other, and by using inducible overexpression and short hairpin RNA-mediated knockdown strategies we demonstrate that hAda3 stabilizes p53 protein by promoting its acetylation. Use of a p53 mutant with mutations of known p300/CREB-binding protein acetylation sites demonstrated that hAda3-dependent acetylation is required for increase in p53 stability and target gene induction. Importantly, we demonstrate that endogenous hAda3 is essential for DNA damage-induced acetylation and stabilization of p53 as well as p53 target gene induction. Overall, our results establish hAda3, a component of coactivator complexes that include histone acetyltransferase p300/CREB-binding protein, as a critical mediator of acetylation-dependent stabilization and activation of p53 upon genotoxic stress in mammalian cells.

Although p53 was identified as a tumor suppressor protein more than 2 decades ago and is the most commonly mutated gene in human cancers (1–3), mechanisms of its tumor suppressor function remain a challenge. The p53 protein is maintained at a very low level in unstressed cells and is rapidly stabilized and activated during periods of cellular stress, such as that induced by DNA damage, resulting in enhanced binding to promoters of specific target genes and their transcriptional activation (4–7). DNA damage-induced post-translational modifications, such as phosphorylation, ubiquitination, and acetylation, have emerged as critical mediators of altered p53 protein stability (4, 8). Acetylation of p53 provides a particularly important mechanism to control p53 stability as well as its activity (5, 9). Acetylation of p53 involves the recruitment of coactivator complexes containing histone acetyltransferases (HATs)[3] to promoter-bound p53 function, a process that concurrently controls the level of histone acetylation and hence the accessibility of active promoters to general transcriptional machinery (10, 11). Understanding the molecular mechanisms that promote stress-induced acetylation and consequent alterations in the activity of p53 is therefore of significant biological interest and extremely relevant to human cancer.

Previous studies have identified a number of HATs that can acetylate p53 protein *in vitro* and appear to mediate p53 acetylation *in vivo*. These analyses have identified p300/CREB-binding protein (CBP) as well as p300/CBP-associated factor (PCAF) as p53-directed HATs (5, 12, 13). Interestingly, different HATs may selectively acetylate specific sites in p53, resulting in distinct biological outcomes. For example, p300-dependent acetylation of Lys-370, -372, -373, -381, and -382 has been correlated with p53 stabilization and increased DNA binding (12, 14, 15). Notably, analysis of knock-in mice with a missense (K317R) mutation suggests a negative regulatory role of the PCAF-dependent Lys-317 (human p53 Lys-320) acetylation site in p53 (16). Thus, cellular factors that facilitate HAT-dependent p53 acetylation are of substantial interest in our understanding of p53 as a tumor suppressor.

We and others have recently identified human Ada3 (alteration/deficiency in activation) as a novel p53-binding protein (17–19). Human Ada3 (hAda3) is the homologue of the yeast Ada3, an essential component of the Ada transcriptional coactivator complex composed of Ada2, Ada3, and Gcn5 (general control nonrepressed 5), a HAT (20). Notably, yeast Ada3, Ada2, and Gcn5 were each required for the transcriptional activity of a human p53 transactivation domain fused to Gal4 DNA-binding domain when expressed in yeast (21). In mammalian cells, overexpression of hAda3 increased p53 levels (17) and enhanced the transcriptional activation of p53 target genes

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[3] The abbreviations used are: HAT, histone acetyltransferase; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; PCAF, p300/CBP-associated factor; hAda3, human Ada3; hGcn5, human Gcn5; GFP, green fluorescent protein; RT, reverse transcription; RNAi, RNA interference; HRP, horseradish peroxidase; shRNA, short hairpin RNA.

(17, 18). Recent biochemical fractionation of mammalian cell extracts identified hAda3 as a component of complexes that include hAda2 and hGcn5 (22). In addition, hAda3 is also found in a number of alternate complexes, such as STAGA complex, that include both hGcn5 and PCAF (23). Recent studies have also shown that hAda3 interacts with p300 (18). Given the ability of Ada3 to interact with transcriptional activation domains, these studies strongly suggest that Ada3 may play a key role to link HAT-containing complexes to p53 and thereby regulate p53 acetylation, stability, and activity.

In this report, we used inducible ectopic hAda3 expression, shRNA-mediated knockdown of endogenous hAda3, and a p53 mutant defective in p300-mediated acetylation to demonstrate an essential role of hAda3 in p53 acetylation, stability, and transcriptional activation following DNA damage.

EXPERIMENTAL PROCEDURES

Cells and Media—H1299, a p53-negative human lung cancer cell line, was grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. 76NTERT, a p53-positive immortalized human mammary epithelial cells line, was grown in DFCI-1 medium, as described earlier (24).

Antibodies—Generation of anti-hAda3 rabbit polyclonal antiserum has been described previously (17). Biotinylation of anti-hAda3 rabbit polyclonal antibody was carried out using EZ-Link NHS-LC-Biotin from Pierce according to the manufacturer's instructions. Monoclonal antibodies against p53 (DO1), p21, and GFP were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), anti-acetylated p53 antibody was obtained from Upstate (Temecula, CA), anti-FLAG antibodies were from Sigma, β-actin antibodies were from Abcam Inc. (Cambridge, MA), and anti-phospho-p53 (Ser-15) was from Cell Signaling Technology Inc. (Boston, MA).

Transient Transfection and Western Blotting—For transfection experiments, H1299 cells were plated in 60- or 100-mm dishes and transfected with the indicated expression constructs (see figure legends) using Lipofectamine 2000 reagent (Invitrogen). The medium was changed after 16 h of transfection, the cells were lysed 40 h post-transfection in lysis buffer (50 mM Tris·Cl, 400 mM NaCl, 0.2% Nonidet P-40, 10% glycerol, and protease inhibitors from Roche Applied Science (Mannheim, Germany), and equivalent amounts of whole cell extracts were processed for SDS-PAGE and subjected to immunoblotting with the appropriate antibodies.

Inducible Expression of hADA3—Retroviral supernatants were produced by co-transfecting the retroviral expression construct pRevTet-ON (BD Biosciences, San Jose, CA) with pIK packaging vector into TSA 54 cells using the calcium phosphate coprecipitation method (19, 25). 76N TERT cells were infected with these retroviral supernatants and selected for 10 days in growth medium containing 150 μg/ml G418. 76NTERT Tet-ON stable cells (5×10^5 cells in a 100-mm dish) were transiently transfected with pRev-TRE-FLAG-Ada3 plasmid, which was generated by cloning FLAG-Ada3 (17) into the HindIII and HpaI sites of pRev TRE vector (BD Biosciences). 24 h after transfection, cells were equally split into three 100-mm dishes. 16 h after plating, cells were either mock-treated or treated with 0.5 μg/ml doxycyclin for 4 or 20 h. Equal amounts of whole cell extracts were subjected to SDS-PAGE followed by Western blotting with anti-FLAG, anti-p53, or anti-β-actin antibodies.

Generation of Stable hADA3 shRNA Knock-down Cells—The hAda3-specific RNAi sequences used in shRNA constructs are as follows: 1, GGTGACAGACGATTCCTGA; 2, GCAATCAGAACAAGCCCTT. The oligonucleotides were cloned in the pSUPER-Retro vector (OligoEngine, Seattle, WA). 76NTERT cells were infected with hAda3 RNAi retroviral supernatants as described previously (19, 25). Virally transduced cells were selected in 0.5 μg/ml puromycin for 3 days, and expression of endogenous hAda3 was assessed in the whole cell lysate using Western blotting using anti-Ada3 polyclonal antibody generated in our laboratory (17). The inducible shRNA constructs were generated by cloning the oligonucleotides (I, GCAATCAGAACAAGCCCTT; II, TCAGAACAAGCCCTTCAGT) in the pSUPERIOR-Retro vector as recommended by the manufacturer (OligoEngine). Commercially available trex-U2OS cells (Invitrogen) were used to express the inducible RNAi constructs, since these cells express the compatible tetracyline regulator.

Co-immunoprecipitation Analyses—Cell lysates were prepared with NETN buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 7.8, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail from Roche). Lysates were precleared with 15 μl of protein A and 15 μl of protein G beads (50% slurry) for 1 h at 4 °C. For immunoprecipitation, 2 mg of precleared supernatant was incubated with 2 μg of antibody for 2 h with occasional rocking. Next, 20 μl of protein A or protein G beads (50% slurry) were added to the above samples and rocked for an additional 1 h at 4 °C. The beads were washed five times with the binding buffer, and the bound proteins were eluted by rocking the beads with the elution buffer (20 mM Tris, pH 7.5, 0.5 M NaCl, 0.1% SDS) for 30 min at 4 °C. Beads were spun down, and the clear supernatants were boiled with the sample buffer followed by SDS-PAGE and immunoblotting with appropriate antibodies.

In Vivo Ubiquitination Assay—H1299 cells were transfected with FLAG-ubiquitin, p53, Mdm2, and GFP-Ada3 expression plasmids as indicated in the figure, using Lipofectamine 2000 (Invitrogen). Forty hours after transfection, cells were treated with MG132 (10 μM) for 6 h, and cell lysates were prepared with lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Nonidet P-40, 0.1% SDS, 1× protease inhibitor mixture (Roche Applied Science), and 5 μM *N*-ethylmaleimide. 1-mg extracts were subjected to immunoprecipitation with anti-p53 (DO-1) antibody, and bound proteins were subjected to Western blot assay using anti-FLAG-HRP antibody.

RNA Extraction and RT-PCR—The total RNA was extracted using Trizol Reagent (Invitrogen). The semiquantitative RT-PCR was performed with 200 ng of total RNA using the following fprimers: p53 forward primer (5′-CAGCCAAGTCTGTGACTTGCACGTAC-3′) and reverse primer (5′-CTATGTCGAAAAGTGTTTCTGTCATC-3′); p21 forward primer (5′-GTGAGCGATGGAACTTCGACTT-3′) and reverse primer (5′-GGCGTTTGGAGTGGTAGAAATC-3′); and glyceraldehyde-3-phosphate dehydrogenase forward primer (5′-ACCTGACCTGCCGTCTAGAA-3′) and reverse primer

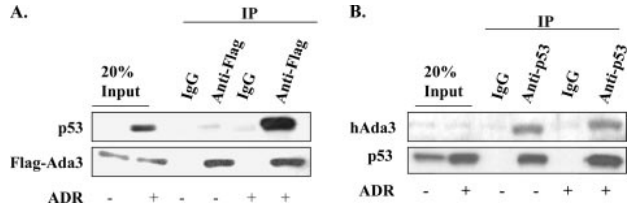

FIGURE 1. **Endogenous interaction of hAda3 with p53.** *A*, U2OS cells stably expressing FLAG-Ada3 were treated with 0.5 μg/ml of adriamycin (*ADR*) or Me_2SO. The cells were lysed in NETN buffer and 2 mg of lysates were immunoprecipitated (*IP*) with either IgG or anti-FLAG antibody. The bound proteins were subjected to Western blotting analysis with anti-p53-HRP or anti-FLAG antibodies. *B*, MCF7 cells were treated with adriamycin as above, and lysates were immunoprecipitated with either anti-p53 (DO-1) antiserum or anti-mouse IgG followed by Western blotting with biotinylated anti-hAda3 polyclonal antibody and streptavidin-HRP secondary reagent.

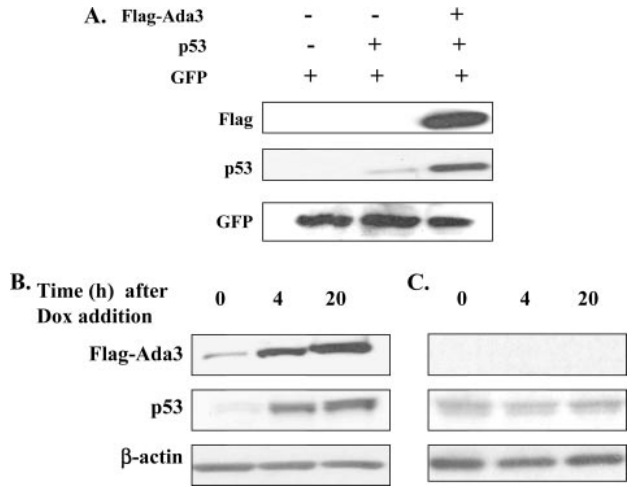

FIGURE 2. **hAda3 overexpression promotes stability of endogenous p53.** *A*, H1299 (p53-null) cells were transfected with pCMV-p53 alone or with pCR3.1 FLAG-tagged hAda3 using Fugene6. GFP was transfected as a transfection control. Cells were lysed, and 20 μg of protein was subjected to Western blotting using anti-FLAG, anti-p53, or anti-GFP antibodies. *B*, 76NTERT Tet-ON stable cells (5×10^5 cells in a 100-mm dish) were transiently transfected with pRev-TRE-FLAG-ADA3 or pRev-TRE vector alone (*C*). 24 h after transfection, cells were equally split into three 100-mm dishes. 16 h after plating, cells were either mock-treated or treated with 0.5 μg/ml of doxycyclin (*Dox*) for 4 or 20 h. Equal amounts of whole cell extracts were subjected to SDS-PAGE followed by Western blotting with anti-FLAG, anti-p53, or anti-β-actin antibodies.

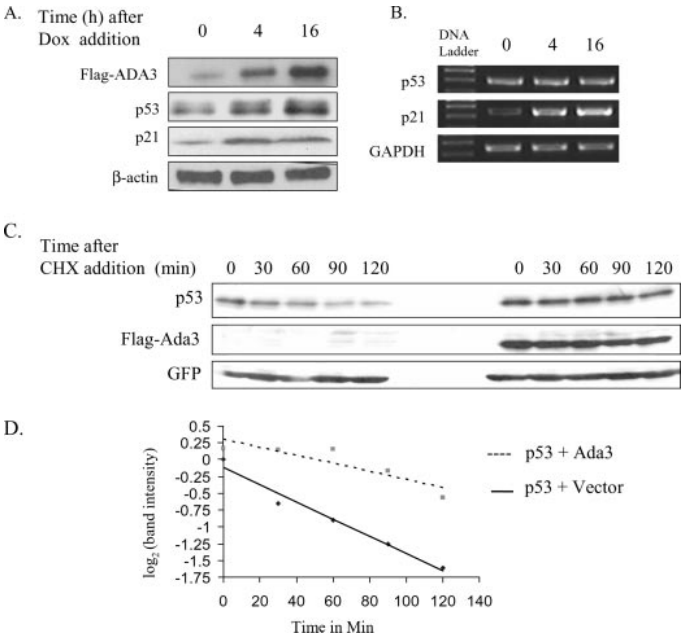

FIGURE 3. **hAda3 stabilizes p53 protein at the post-transcriptional level.** 76NTERT Tet-ON stable cells were transiently transfected with pRev-TRE-FLAG-Ada3. Cells were then equally split into three 100-mm dishes and either mock-treated or treated with 0.5 μg/ml of doxycyclin for 4 or 16 h. Cells were then harvested for protein or mRNA analysis. Equal amounts of whole cell extracts were subjected to SDS-PAGE followed by Western blotting (*WB*) using anti-FLAG, anti-p53, or anti-β-actin antibodies (*A*). Total RNAs were analyzed by RT-PCR to detect the levels of p53, p21, or glyceraldehyde-3-phosphate dehydrogenase (*B*). *C*, hAda3 overexpression extends p53 half-life. H1299 cells were transfected with either pCMV-p53 and pCR3.1 vector or pCMV-p53 along with pCR3.1-FLAG hAda3. GFP was transfected in each set as a transfection control. Twenty-four hours after transfection, each set of cells was replated into five equal parts. After 16 h of seeding, cells were treated with cycloheximide (50 μg/ml) and harvested at the indicated time intervals. Cell lysates were analyzed by Western blotting using anti-p53, anti-FLAG, or anti-GFP antibodies. The intensity of p53 bands were quantified by densitometry against GFP using ScionImage software and plotted against time of cycloheximide treatment (*D*). Each decrease of 1 unit of log 2 is equivalent to one half-life.

(5′-TCCACCACCCTGTTGCTGTA-3′). The RT-PCR products were visualized under UV after running on an ethidium bromide-stained 2% agarose gel.

Analysis of the p53 Protein Turnover—Two 100-mm dishes of H1299 cells were transfected with plasmids expressing p53 alone or in combination with FLAG-hAda3. At 16 h post-transfection, cells were trypsinized and pooled, and equal numbers of cells were replated on five 60-mm plates. After, 16 h, 50 μg/ml of cycloheximide (Sigma) was added to the medium, and cells were harvested at the indicated time points. Total cell extracts were prepared, and equivalent amounts were run on SDS-PAGE and analyzed by Western blotting. Densitometric analysis was carried out on scanned images using ScionImage for Windows software (Scion Corp., Frederick, MD).

Analysis of Cells Exposed to DNA Damage—76NTERT cells stably expressing scrambled RNAi or hAda3 RNAi were either exposed to 20 grays of ionizing radiation or treated with 2.5 μg/ml actinomycin D or 0.5 μg/ml adriamycin; cells were harvested at 0, 4, or 8 h of treatments; and lysates were prepared as above and immunoblotted for acetylated p53, total p53, p21, or β-actin proteins.

RESULTS

Endogenous hAda3 Interacts with Endogenous p53—We and others have shown previously (17, 18) that ectopic expression of both hAda3 and p53 showed hAda3 interaction with p53. Here, we confirmed this interaction by expressing FLAG-tagged hAda3 and endogenous p53 in U2OS cells, treating cells with adriamycin and immunoprecipitating hAda3 by anti-FLAG antibody, followed by Western blotting with anti-p53 antibody (Fig. 1*A*). In a second set of experiments, MCF-7 cell lysates after adriamycin treatment were immunoprecipitated with anti-p53 antibody, followed by Western blotting with biotinylated anti-hAda3 polyclonal antibody and streptavidin-HRP.

A clear association of both ectopically expressed hAda3 as well as endogenous hAda3 with endogenous p53 was seen in both cells, and the interaction increased dramatically upon adriamycin treatment (Fig. 1, *A* and *B*). These results support and extend our previous findings of interaction of hAda3 with p53.

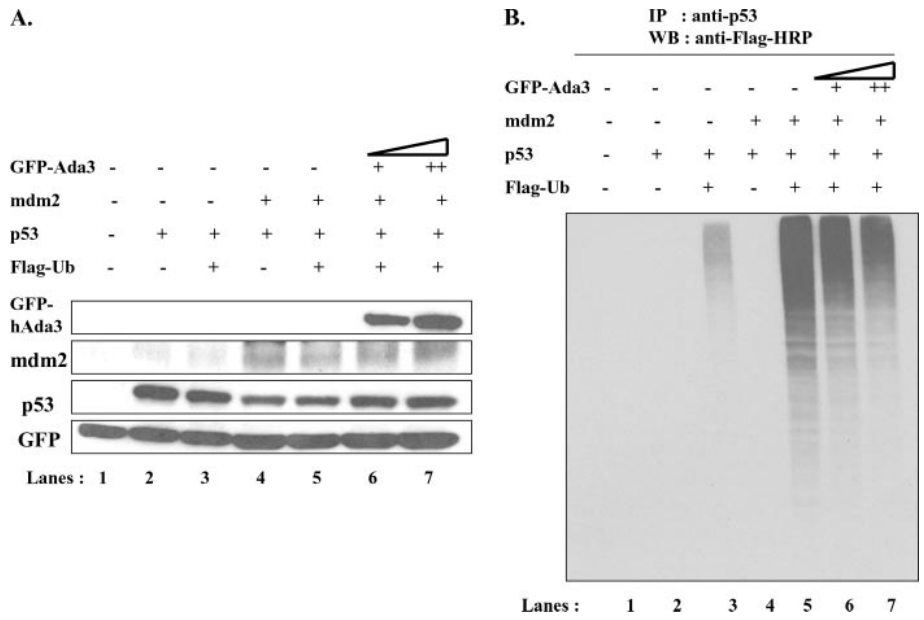

FIGURE 4. **hAda3 reduces the Mdm2-mediated ubiquitination of p53.** H1299 cells were transfected with the indicated combination of plasmids. After 40 h of incubation, cells were treated with MG132 (10 μM) for 6 h. Cell lysates were immunoprecipitated (*IP*) with anti-p53 antibody, and ubiquitination was assayed by probing with anti-FLAG-HRP antibody (*B*). Expression of the transfected constructs were analyzed by immunoblotting (*WB*) equal amounts of cell lysates with GFP, Mdm2, or p53 antibodies (*A*).

Ectopic hAda3 Overexpression Induces the Stabilization of Endogenous p53— hAda3 binds to p53, and that overexpression of both hAda3 and p53 enhances p53-mediated transcriptional activity (17, 18). In these studies, we also observed that overexpression of hAda3 increased the levels of a co-introduced ectopic p53 protein (17) (Fig. 2*A*). To assess if hAda3 exerts a similar influence on endogenous p53, we engineered a Tet-ON derivative of an hTERT-immortalized human mammary epithelial cell line (76N-TERT) for Tet-inducible expression of FLAG-hAda3. Expression of FLAG-hAda3 in this cell line was doxycyclin dose- and time-dependent (Fig. 2*B*; data not shown). When hAda3 levels were progressively elevated in these cells by doxycyclin treatment for increasing time periods, a similar progressive increase in the level of endogenous p53 protein was seen (Fig. 2*A*); no change in p53 levels was observed upon doxycyclin treatment of the same cell line transfected with the control vector (Fig. 2*B*). These observations extend our earlier results with ectopic p53 (17) and demonstrate that elevation of the cellular hAda3 protein levels increases the level of the endogenous p53 protein.

Ectopic hAda3 Expression Increases the Stability of p53 Protein—Given the importance of the regulated p53 protein degradation in the normal cells (9), it appeared likely that hAda3-dependent increase in p53 levels may reflect a post-translational mechanism. However, we wanted to first rule out any influence of hAda3 expression on p53 mRNA levels. For this purpose, 76N-TERT Tet-ON cells were transfected with pRev-TRE-FLAG-hAda3 plasmid, followed by doxycyclin induction for various time points. Parallel samples were processed for Western blotting of protein lysates and RT-PCR analysis of p53 message levels. Although doxycyclin induction led to the expected FLAG-Ada3 induction over time and concomitant increased p53 protein levels, RT-PCR analysis showed that p53 mRNA levels remained unchanged similar to mRNA levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase, used as a loading control (Fig. 3, *A* and *B*). In contrast, the increase in the level of p21 protein, a transcriptional target of p53, was accompanied by an expected doxycyclin-dependent increase in p21 mRNA levels (Fig. 3*B*). These observations indicated that the accumulation of p53 protein upon hAda3 overexpression is likely to be due to a post-transcriptional mechanism.

In order to investigate if the hAda3-dependent increase in p53 levels reflected an increase in the stability of p53 protein, we assessed the half-life of p53 protein following cycloheximide block of new protein synthesis. For this purpose, p53-null H1299 cells were transfected with p53 expression vector pCMV-p53 alone or together with PCR 3.1-based FLAG-hADA3 expression vector. The transfected cells were incubated in the presence of cycloheximide, and p53 levels were assessed by immunoblotting of cellular extracts prepared at various times after cycloheximide addition followed by densitometry to assess the half-life. As shown in Fig. 3*C*, the overexpression of FLAG-hAda3 resulted in a slower loss of p53 signals compared with cells without hAda3 overexpression. The half-life of the p53 protein increased from around 68 min in cells without Ada3 overexpression to more than 120 min in cells with hAda3 overexpression (Fig. 3*D*). This finding, together with the lack of any effect on p53 mRNA levels, supports our conclusion that ectopic hAda3 overexpression increases p53 protein levels by promoting its stability.

hAda3 Inhibits the Mdm2-mediated Ubiquitination of p53— The p53 protein is constantly ubiquitinated and is degraded by the ubiquitin-proteasome pathway (26), and Mdm2 is known to play a major role in this process (27, 28). Recent reports have shown that several proteins that directly associate with and stabilize p53 inhibit p53 ubiquitination (29, 30). Thus, we examined if hAda3-induced increase in p53 affects p53 ubiquitination. For this purpose, H1299 cells were transfected with plasmids expressing p53, FLAG-ubiquitin, Mdm2, and increasing amounts of GFP-Ada3. Cells were then treated with 26 S proteasome inhibitor MG132, and extracts were immunoprecipitated with anti-p53 antibody followed by Western blotting with anti-FLAG antibody conjugated to HRP. The same blot was also probed for p53, Mdm2, or GFP-Ada3 (Fig. 4*A*). As expected, coexpression of ubiquitin and Mdm2 significantly enhanced p53 ubiquitination as compared with p53 alone (Fig. 4*B*, compare *lanes 3* and *5*). Notably, increasing amounts of hAda3 dramatically reduced the ubiquitination of p53 in a dose-dependent manner (Fig. 4*B*, compare *lane 5* with *lanes 6* and *7*). Fig. 4*A* is Western blotting of lysates used in Fig. 4*B* with the

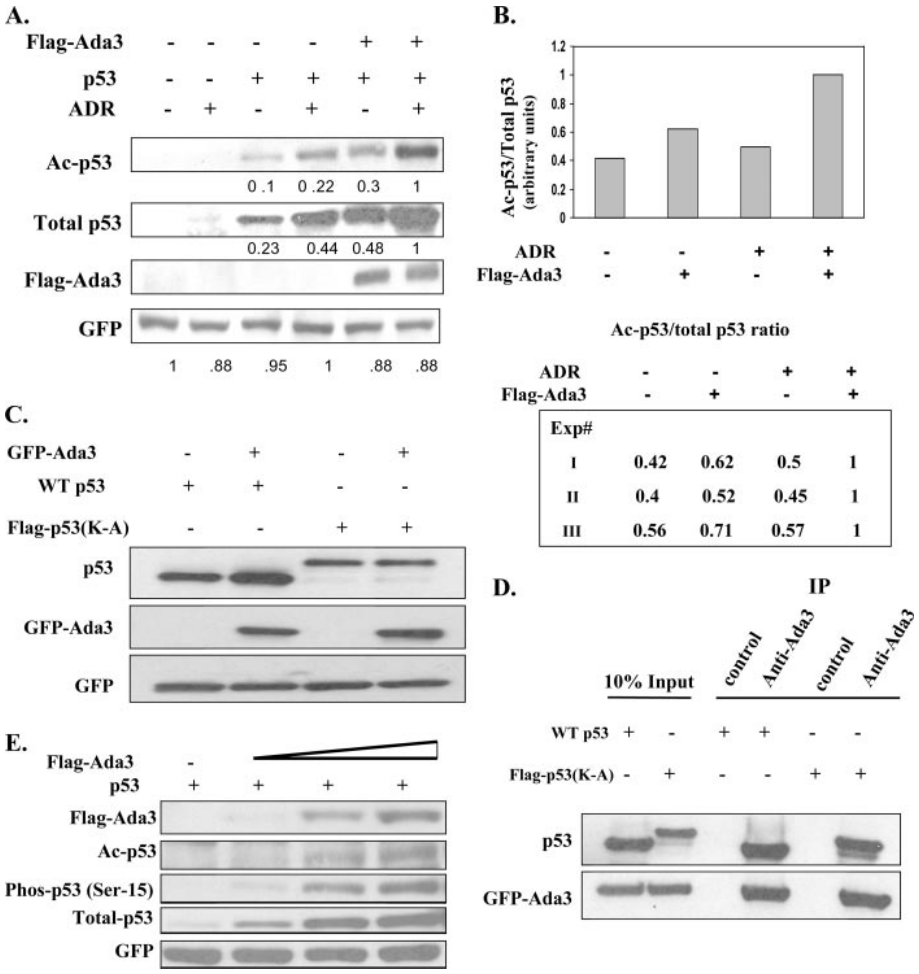

FIGURE 5. **hAda3 promotes p53 acetylation.** *A*, H1299 cells were transfected with either vector, p53, or p53 together with FLAG-hAda3. GFP was transfected in each set as a transfection control. The next day, each set of cells were split into two equal parts. After attachment, cells were either treated with Me_2SO or with adriamycin (*ADR*; 0.5 μg/ml) for 16 h. Cells were harvested, and equivalent amounts of cell lysates were subjected to Western blotting using indicated antibodies. The *numbers below* the acetylated p53, total p53, and GFP bands are the arbitrary values for relative intensity of the respective signals. *B*, intensity of the acetylated p53 and the total p53 signals was quantitated and equalized against GFP using ScionImage software. The *upper panel* shows the histogram plotted with the ratio of acetylated *versus* total p53 with or without Ada3 overexpression and/or adriamycin treatment. Experiments were repeated three times, and the values obtained with densitometry are indicated in the *table below*. A representative experiment is shown in *A* and *B*. *C*, hAda3 overexpression fails to stabilize p53(K-A) mutant defective in p300/CBP acetylation. H1299 cells were transfected with p53 (*WT*) or p53(K-A) mutant alone or together with vector or GFP-Ada3. GFP alone was included in all sets as a transfection control. Cells were harvested after 40 h of transfection, and equivalent amounts of whole cell lysates were subjected to Western blot analysis using anti-p53 or GFP antibodies. *D*, equal amounts of extracts from the above transfection were subjected to immunoprecipitation with control or hAda3 antibodies. The bound proteins were subjected to Western blotting with anti-p53 antibody. *E*, ectopic expression of hAda3 increases p53 phosphorylation. H1299 cells were transfected with 1, 2, and 3 μg of FLAG-hAda3 expression plasmid along with 20 ng of pCMV-p53 plasmid. GFP (0.5 μg) was included in every transfection as a control. Forty hours after transfection, cell lysates were prepared as described under "Experimental Procedures," and equal amounts of cell extracts were subjected to Western blot analysis using FLAG, acetylated-p53, phospho-p53 (Ser-15), or GFP antibodies.

indicated antibodies. These results clearly demonstrate that hAda3 stabilizes p53 by inhibiting its ubiquitination.

hAda3 Overexpression Enhances the Level of p53 Acetylation—Stability of p53 is controlled by post-translational modifications with ubiquitination signaling protein degradation and a number of other modifications, such as acetylation and phosphorylation (4, 5). Acetylation of lysine residues can enhance stability by preventing ubiquitin modification as well as by altering protein-protein and protein-DNA interactions (5, 12, 15, 31). Since hAda3 has been demonstrated to form multiprotein complexes containing HATs, such as Gcn5, p300, and PCAF (22, 32), together with the ability of yeast Ada3 to stimulate histone acetylase activity (32–34), we hypothesized that hAda3 may stabilize p53 by promoting its acetylation. To test this possibility, H1299 cells were transfected with p53 alone or co-transfected with FLAG-hAda3. 16 h after transfection, each transfected plate was split into two plates. One plate in each set was used as a control while the other plate was exposed to adriamycin for 16 h to induce DNA damage, and cell lysates were analyzed for total and acetylated p53 levels using immunoblotting. As expected, total as well as acetylated p53 levels increased (about 2-fold) upon adriamycin treatment (Fig. 5, *A* and *B*). Notably, acetylated p53 levels were higher (3-fold) in cells overexpressing hAda3, and these levels increased further upon adriamycin treatment; the Ada3-dependent increase in acetylated p53 paralleled the increase in total p53 levels (Fig. 5, *A* and *B*). Fig. 5*B* shows the quantification of the data presented in Fig. 5*A* using ScionImage software. These results show that overexpression of hAda3 promotes acetylation of p53 in a statistically significant manner ($p < 0.05$).

Acetylation of p53 Is Required for hAda3-dependent Stabilization—Recent studies have delineated a number of lysine residues on p53 that serve as sites for acetylation by p300/CBP, the major p53-targeted HAT, and PCAF (4, 12, 35). Notably, p300/CBP has been shown to acetylate p53 on Lys-370, Lys-372, Lys-373, Lys-381, and Lys-382, and missense mutations of these lysines result in a mutant that is no longer acetylated by p300 (36). Thus, we used this acetylation-defective mutant to assess if acetylation was required for hAda3-dependent p53 stabilization. H1299 cells were transfected with wild-type or mutant p53 with or without GFP-Ada3, and cell lysates were analyzed using immunoblotting for total and acetylated levels of p53. In contrast to the expected increase in the level of wild-type p53 when hAda3 was co-expressed, the level of mutant p53 protein (which migrates with slower mobility due to the presence of the FLAG tag) was unaltered by the overexpression of hAda3 (Fig. 5*C*).

To rule out the possibility that lack of stabilization of mutant p53 may be due to its inability to bind to hAda3, we carried out

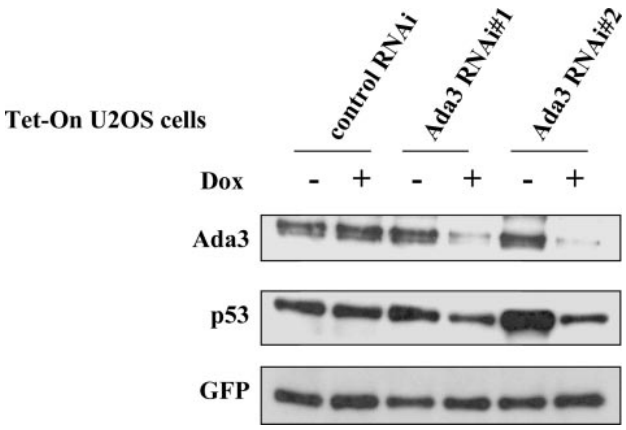

FIGURE 6. **Transient knockdown of hAda3 expression decreases the levels of endogenous p53.** Trex-U2OS cells (Invitrogen) were transfected with either scrambled shRNA or inducible hAda3 shRNA constructs together with GFP. The next day, each set of cells was split into two equal parts and either treated with ethanol or with doxycyclin (*Dox*; 0.5 μg/ml) for 2 days. Cells were harvested, and equivalent amounts of protein from the cell lysates were subjected to Western blotting using anti-p53 or GFP antibodies.

co-immunoprecipitation analyses of the cell lysates analyzed in Fig. 5*C*. Anti-p53 immunoblotting of anti-hAda3 immunoprecipitates demonstrated comparable hAda3 association with the wild-type and mutant p53 (Fig. 5*D*). These results demonstrate that acetylation of p53, apparently via a hAda3-associated HAT(s), is a prerequisite for hAda3-dependent stabilization of p53.

It is well known that DNA damage-induced increase in p53 activity involves a phosphorylation-acetylation cascade (5, 37). In order to investigate the role of hAda3 on phosphorylation of p53, plasmid expressing wild-type p53 was transfected in H1299 cells along with increasing amounts of hAda3. Forty hours after transfection, cell lysates were analyzed for the levels of hAda3, phospho-p53 (Ser-15), acetylated p53, and total p53 by Western blotting. As shown in Fig. 5*E*, hAda3 induced phosphorylation of p53 in a dose-dependent manner. This finding indicates that hAda3 also affects the p53 phosphorylation at the serine 15 residue.

RNAi-mediated Depletion of hAda3 Reduces the Levels of Endogenous p53—Collective evidence presented above using hAda3 overexpression analyses in the context of both ectopically expressed and endogenous p53 raised the possibility that endogenous hAda3 could play a physiological role in stabilizing p53. To test this possibility, we used a Tet-ON derivative of the U2OS cell line with wild-type p53 to knock down the expression of endogenous hAda3. These cells were transfected with shRNA constructs encoding either a scrambled shRNA or two distinct hAda3-inducible shRNA constructs, together with GFP (as a transfection control). Following transfection, the cells were either treated with ethanol (solvent) or with doxycyclin to induce the shRNA expression, and cell lysates were analyzed by immunoblotting with the indicated antibodies. Anti-Ada3 immunoblotting showed that doxycyclin induction of cells transfected with scrambled shRNA had no effect on the levels of hAda3 and that p53 levels remained unchanged (Fig. 6). In contrast, doxycyclin induction of cells transfected with either hAda3 shRNA resulted in substantial reduction in Ada3 protein levels; concomitantly, we observed a decrease in the levels of endogenous p53 protein in cells where hAda3 shRNA was induced (Fig. 6). These results indicated that endogenous hAda3 is required for the stability of p53 protein.

Endogenous hAda3 Is Required for Damaged DNA-induced Acetylation of Endogenous p53—Given the important role of p53 during DNA damage response, we wished to assess the role of endogenous hAda3 in p53 acetylation upon DNA damage. We thus examined the levels of total and acetylated p53 in cells with or without hAda3 knockdown when exposed to DNA damage. For this purpose, we generated stable derivatives of the 76NTERT cell line in which the two independent hAda3 shRNAs described above or a scrambled shRNA control were introduced via retrovirus infection followed by puromycin selection. The cells used in these experiments show an expected decrease in Ada3 levels and consequently decreased levels of p53 protein (Fig. 7*A*). These cells were either left untreated or exposed to different DNA-damaging conditions (actinomycin D, adriamycin, or ionizing irradiation). Western blot analysis of total cell lysates demonstrated that hAda3 shRNAs but not the scrambled shRNA resulted in a marked reduction in hAda3 protein levels (Fig. 7, *B–D*). Notably, immunoblotting with antibodies specific for acetylated Lys-373 and Lys-382 in p53 revealed that the DNA damage-induced increase in p53 acetylation as well as total p53 protein levels were drastically reduced in the hAda3 knockdown cells as compared with the control cells (Fig. 7, *B–D*). Concurrent immunoblot analysis demonstrated the expected decreased levels of p53 target p21 in hAda3 knockdown cells as compared with control cells, indicating that hAda3-dependent acetylation and stabilization of p53 is required for the activity of p53 as a transcriptional activator. Next, we wanted to find out if hAda3 has any effect on the levels of p21. H1299 cells were transfected with increasing amounts of FLAG-hAda3 expression plasmid. Western blot analysis of the transfected cell extracts clearly showed that the p21 level remains unaffected by increasing expression of hAda3 (Fig. 7*E*). This observation shows that p21 expression is dependent on hAda3-induced p53 stabilization. Taken together, these results demonstrate that hAda3 plays an essential role in p53 acetylation and stabilization of endogenous p53 protein and its consequent activation as a transcription factor.

DISCUSSION

The p53 gene is the single most frequently inactivated gene in human cancers and is mutated in ~50% of all cancers (1–3). The p53 protein is a sequence-specific homotetrameric transcription factor that becomes activated in response to many forms of cellular stress, such as irradiation, hypoxia, drug-induced DNA damage, and oncogene activation; in response, p53 orchestrates the transcription of many genes to either arrest cell proliferation or, more dramatically, to induce apoptosis (38, 39). A major early step in the activation of p53-mediated cellular responses involves the stabilization and activation of the DNA binding activity of p53. Post-translational modifications have emerged as key mechanisms in these early events (4). Cellular factors that control the post-translational modifications of p53 and hence its functional states have therefore become increasingly important in understanding the regulation of p53. Here, we demonstrate a critical role of hAda3 in DNA damage-

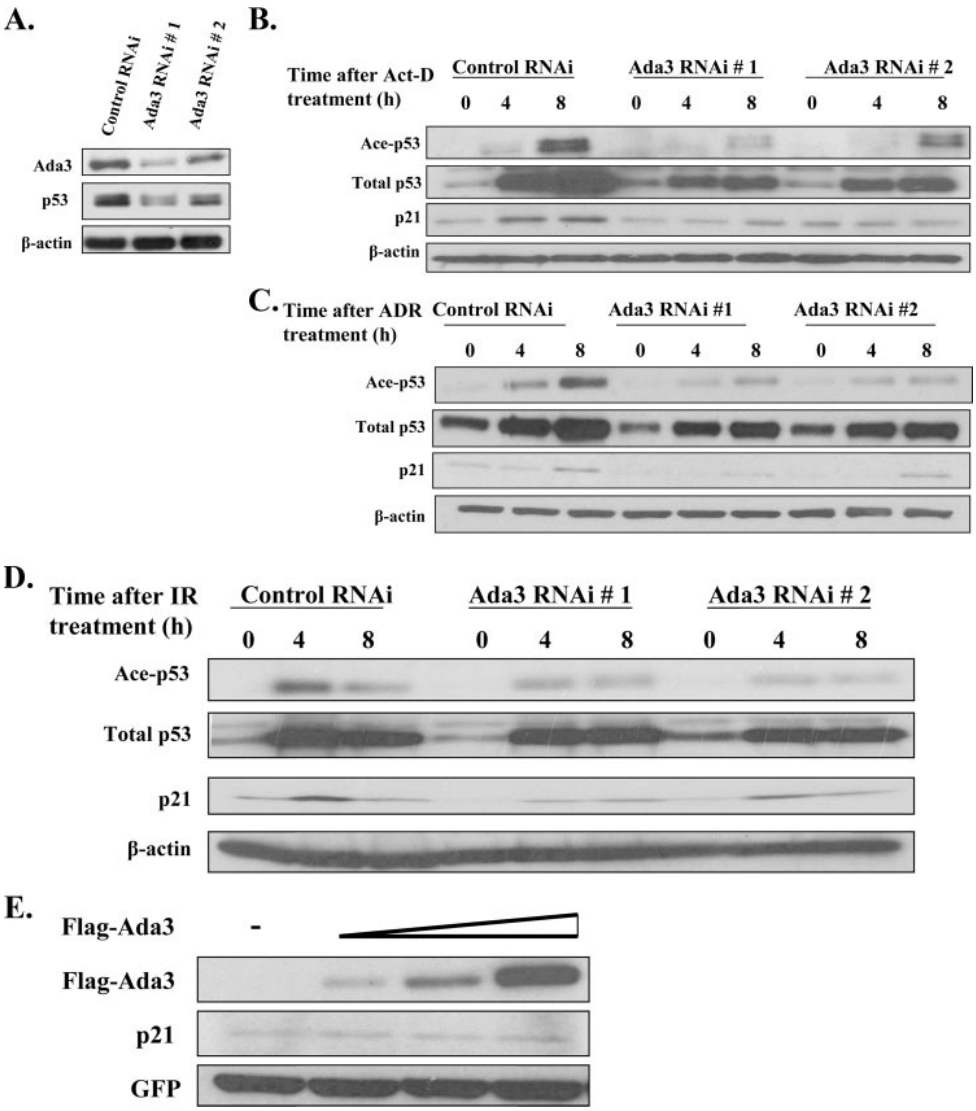

FIGURE 7. **shRNA-mediated knockdown of endogenous hAda3 dramatically reduces the levels of acetylated p53 upon DNA damage.** *A*, levels of hAda3 and p53 proteins in 76N TERT cells stably expressing scrambled shRNA and two independent shRNA against hAda3 as described under "Experimental Procedures." These cells were either left untreated or treated with actinomycin D (*B*) or adriamycin (*C*) or exposed to ionizing radiation (*D*) for 4 or 8 h. Cells were then harvested, and equivalent amounts of total cell lysates were subjected to Western blot analysis using the indicated antibodies. *E*, H1299 cells were transfected with 2, 3, and 6 μg of FLAG-hAda3 expression plasmid. GFP (0.5 μg) was included in every transfection as a control. Forty hours after transfection, cell lysates were prepared as described under "Experimental Procedures," and equal amounts of cell extracts were subjected to Western blot analysis using FLAG, p21, or GFP antibodies.

induced stabilization and activation of p53. We also provide evidence that hAda3-dependent regulation of p53 stability and activity is mediated by acetylation of p53.

Ada3 was initially identified as a core component of the yeast Ada coactivator complex that includes an adaptor protein Ada2, which in turn associates with a HAT protein Gcn5 (34). Ada3 interacts directly with the activation domains of transcriptional complexes, thus serving as a key component in the recruitment of Ada complex and its associated HAT activity to transcriptional activators bound to specific promoters (32, 40, 41). Recent studies of Ada3 and other Ada components in higher eukaryotes, including mammals, have demonstrated that these proteins assemble into multiple complexes that can vary significantly from the trimolecular yeast Ada complex. For example, mammalian cells appear to contain a Gcn5 complex that does not include Ada2 (42). Similarly, two distinct Ada2 proteins exist in higher eukaryotes, and studies in *Drosophila* demonstrate that both Ada2 proteins are essential for development, indicative of distinct functions (43). In general, however, Ada3 has been found as an invariant component of many Ada-related complexes in mammals and other higher eukaryotes, indicating a pivotal role of Ada3 in coactivator functions of these complexes. In addition, Ada3 emerged as a binding partner of the human papilloma virus E6 oncoprotein (17, 19, 25), suggesting the possibility that viral oncoproteins alter the function of Ada3 during oncogenesis. Thus, we have focused our efforts on understanding the functional roles of human Ada3.

Recently, we and others have observed that hAda3 associates with p53 and that hAda3 overexpression increases the transcriptional activity of p53 (17, 18). However, the physiological implications of this interaction and the mechanism(s) by which hAda3 regulates p53 function have remained unclear. Studies presented here addressed these issues and establish hAda3 as a physiologically relevant and apparently major regulator of p53 stability and activity via the promotion of p53 acetylation. In general, the function of Ada complex, similar to other HAT-containing coactivator complexes, has been examined in the context of chromatin modification through histone acetylation. Recent studies, however, have also demonstrated that proteins involved in transcriptional regulation, including transcription factors themselves, are also modified by acetylation. Specifically, acetylation of p53 provides a crucial mechanism to control its activity. It has been demonstrated that DNA damage and other stress stimuli rapidly induce p53 acetylation on a number of lysine residues, and two HATs, p300/CBP and PCAF, have been implicated as principal participants in the acetylation process (5, 12, 44), although their relative role as well as the role of Gcn5 and other HATs has not been carefully delineated. Importantly, mutation of p53 on sites identified as p300/CBP acetylation sites abrogates DNA damage-induced activation of p53 (36). Knock-in mutations of acetylation sites in the mouse further confirm the role of acetylation in fine tuning of p53-mediated DNA damage response *in vivo* (16). Thus, our finding that hAda3 plays a major role in

acetylation-dependent stabilization and activity of p53 represents a significant step forward in understanding the mechanisms that control p53 function. Notably, previous analyses using human p53 transactivation domain fused to Gal4 DNA-binding domain showed that yeast Ada3, Ada2, and Gcn5 were all required for transactivation in the yeast (21). These findings suggest that the role of Ada3 to recruit histone acetylation machinery for acetylation of transcription factors and the associated factors may be evolutionarily conserved. Further analyses in the context of other Ada3-interacting transcriptional regulators as well as studies in nonmammalian species should help address if this is indeed the case.

As HAT-dependent acetylation occurs on lysine residues, which are also targeted by ubiquitin modification, one mechanism for acetylation-dependent regulation of p53 is its antagonism of ubiquitin-dependent degradation. Ubiquitin ligases, in particular Mdm2, physically interact with p53 and target it for ubiquitin-dependent proteasomal degradation, ensuring low p53 levels in unstressed cells. Our *in vivo* ubiquitination assay shows that hAda3 interferes with the Mdm2-mediated ubiquitination of p53, which further adds up to the p53 stabilization potential of this adapter protein. Ubiquitin modification has also been shown to promote nuclear export of p53, further ensuring its inactive state (14). Upon DNA damage and other stresses, Mdm2 association decreases, resulting in stabilization of p53 and reduction of its nuclear export and availability for binding to target promoters. It is noteworthy that Mdm2 and p300/CBP compete for binding to the transcriptional activation region of p53 (45), providing a potential switch between ubiquitination and acetylation. We speculate that Ada3 interaction with p53 provides a mechanism whereby p300/CBP as well as other Ada3-associated HATs are juxtaposed to p53 for its acetylation-dependent stabilization. Our finding that a mutant p53 that cannot be acetylated on major p300/CBP acetylation sites is not stabilized by Ada3 is consistent with this idea. Aside from antagonism of ubiquitination, acetylation also plays a positive role in promoting DNA-binding activity of p53 by promoting tetramerization and possibly by other unknown mechanisms (5, 36). The relative contribution of Ada3-dependent acetylation of p53 *versus* histone acetylation at p53-bound promoters is an important question that will need in depth future analyses.

The substantial degree of inhibition of p53 acetylation, stabilization, and target gene induction by Ada3 knockdown indicates a relatively important contribution of this protein in recruiting HAT machinery to p53. This crucial role for Ada3 could reflect a predominant role of a HAT that is critically dependent on Ada3 for association with p53 or the alternative possibility that most p53-directed HATs require Ada3. Gcn5 and PCAF are known to be in complex with Ada3, and other studies indicate that Ada3 and p300/CBP may also be in a single complex (18, 22, 23). These findings are consistent with the latter proposal. However, it remains possible that Ada3 may not recruit the various p53-dependent HATs universally. Previous studies of p300/CBP indicate a potentially direct association with p53 (35); however, these studies were not carried out using pure proteins, leaving open the possibility that p300/CBP may be recruited to p53 via Ada3. Further biochemical analyses and availability of cells and mice deficient in Ada3 should help to examine these models. These studies are under way in our laboratory.

In conclusion, our identification of hAda3 as a critical regulator of p53 acetylation, stability, and activity provides a new insight into p53 biology. Further studies of the components of Ada3-containing coactivator complexes as well as biological analyses of animals deficient in Ada complex components and p53-directed HATs should help delineate the biochemical basis as well as the overall biological importance of Ada3-dependent p53 regulation.

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An Essential Role of Human Ada3 in p53 Acetylation

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