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Research Article

Regulation of turnover of tumor suppressor p53 and cell growth by E6-AP, a ubiquitin protein ligase mutated in Angelman mental retardation syndrome

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Abstract. E6-AP is a founding member of HECT (homologous to E6-AP C terminus) domain subfamily of E3 ubiquitin ligases. It degrades tumor suppressor p53 in association with the E6 oncoprotein of the human papilloma virus. However, there are conflicting reports on its role in the degradation of p53 in the absence of E6 oncoprotein. Here, we studied the role of E6-AP in regulation of p53 in mouse neuro 2a cells. Overexpression of E6-AP in neuro 2a cells increased the ubiquitylation and degradation of p53, which could be prevented upon deletion of HECT domain. E6-AP also directly ubiquitylated p53 in an *in vitro* ubiquitylation assay. Partial knockdown of E6-AP increased the levels of p53 and p53-dependent transcription. Partial knockdown also increased neuronal cell death, which may be mediated partly *via* p53. Our result suggests that E6-AP not only enhances the degradation of p53 but also regulates the neuronal cell growth.

Keywords. E6-AP, p53, Angelman syndrome, transcription, cell growth, apoptosis.

Introduction

Angelman syndrome (AS) is a neurodevelopmental disorder characterized by severe mental retardation, lack of speech, ataxia, abnormal gait, seizures, easily provoked smiling and laughter and sleep disturbances [1, 2]. The incidence of AS is 1 in 15 000–20 000 births [3, 4]. The disease is caused primarily by maternal deletion of chromosome 15q11-q13, which encompasses the *UBE3A* locus [3], and also by paternal uniparental disomy of chromosome 15 [5], imprinting defects [6, 7] or loss of function mutation of the *UBE3A* gene [8–11]. All of these mechanisms lead to an absence of functional copy of the *UBE3A* gene. Although the deletion of other genes in chromosome 15q11-q13 may also be associated to AS, the identification of loss of function mutation in *UBE3A* gene in AS patients provides the most compelling evidence that the disease is caused primarily by a maternal deficiency of *UBE3A* gene.

The *UBE3A* gene encodes E6-AP ubiquitin protein ligase, an enzyme involved in the intracellular protein degradation through the ubiquitin proteasome system (UPS). E6-AP was discovered based of its ability to promote the ubiquitin-mediated degradation of tumor suppressor p53 in association with the E6 oncoprotein of the human papilloma virus (HPV) [12, 13]. The degradation of a protein through UPS involves two distinct and successive steps: protein ubiquitylation

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and the degradation of the ubiquitylated proteins by the 26S proteasome [14]. The ubiquitylation of protein substrates is carried out by a series cellular enzymes known as E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin protein ligase), resulting in multiubiquitylation of protein. A target protein must be tagged with a multiubiquitin chain composed of at least four ubiquitins before it can be recognized and degraded by the proteasome. The ubiquitin-mediated protein degradation plays a very important role in numerous critical cellular events including regulation of the cell cycle, signal transduction, antigen processing, synaptic plasticity, and transcription [14].

E6-AP is the founding member of HECT (homologous to E6-AP C terminus) domain family of E3 ubiquitin ligases that provides specific recognition of substrate protein [15, 16]. It is therefore hypothesized that the AS phenotype might be caused by failure of ubiquitylation and subsequent degradation of a variety of target substrates of E6-AP. Several recent reports showed that many AS-associated point mutations are actually located in the HECT domain and may affect the ubiquitin ligase activity [17]. The substrates of E6-AP so far known includes a human homologue of the yeast DNA repair protein RAD23 [18], the multicopy maintenance protein 7 subunit involved in the initiation of DNA replication [19], Src family tyrosine kinase [20], epithelial cell transforming sequence-2 oncogene [21] and E6-AP, which is a target for itself [22]. In addition to its ubiquitylation activity, E6-AP can also serve as a transcriptional coactivator for steroid hormone receptors [23]. The transcriptional coactivator function of E6-AP is mediated by the N-terminal domain and seems to be independent of its ubiquitin ligase activity. Unfortunately, identification of these target proteins or transcriptional coactivator function has yet to shed light on any possible mechanism underling AS pathogenesis. Although E6-AP is able to ubiquitylate p53 in association with E6 oncoprotein, there are conflicting data regarding its ability to interact with p53 in the absence of E6, and no physiological significance of interaction between E6-AP and p53 in the absence of viral protein is known [24–26]. However, elevated levels of p53 have been demonstrated in the brain and prostate gland of E6-AP null mice [27, 28]. This suggests that E6-AP is possibly involved in the regulation of p53 in HPV-negative cells; however, the underline mechanism is not known. Surprisingly, another mouse model of AS generated by Miura et al. [29] did not show any sign of p53 elevation in the brain. Therefore, the role of E6-AP in the ubiquitylation and subsequent degradation of p53 in the absence of E6 oncoprotein is still unclear. In the present study, we investigated the regulation of p53 by E6-AP in the neuronal cells. We found that E6-AP is not only involved in the ubiquitylation and degradation of p53 but also regulates cell growth.

Materials and methods

Materials. MG132, cycloheximide, TRIzol reagent, pifithrin-α, N^6,2′-*O*-dibutyryl-adenosine-3′,5′-cyclic monophosphate (dbcAMP), all *in vitro* ubiquitylation assay reagents, rabbit polyclonal anti-ubiquitin, mouse monoclonal anti-β-tubulin and all cell culture reagents were obtained from Sigma. Lipofactamine® 2000, Probond purification system for His-tagged proteins and Block-IT™ Pol II miRNA RNAi expression vector kit were purchased from Invitrogen. RT-PCR kits were purchased from Invitrogen and TaKaRa Biomedicals. Luciferase reporter gene assay kit, *In situ* cell death detection kit, and protein G agarose beads were purchased from Roche. Mouse monoclonal anti-E6-AP and anti-V5 were purchased from BD Pharmingen and Invitrogen, respectively. Rabbit polyclonal anti-bax, anti-p21 and anti-p53 and mouse monoclonal anti-parkin were from Santa Cruz Biotechnology. Goat anti-rabbit IgG-rhodamine, goat anti-mouse IgG-FITC, alkaline phosphatase-conjugated anti-mouse and anti-rabbit IgG were purchased from Vector Laboratories.

Expression plasmids. The full-length E6-AP cDNA was isolated from neuro 2a cell total RNA using an RT-PCR kit (TaKaRa Biomedicals) and then cloning the PCR product into pcDNA3.1 TOPO TA cloning vector. Primer sequences were 5′-ACCATGGAGCGAGCAGCTGCAAAG CATCT-3′ (sense) and 5′-GTACAGCATGCCAAATCCTTTGGC-3′ (antisense). The HECT domain-deleted construct (amino acids 1–413) was made using PCR and subcloning the PCR product into pcDNA3.1 TOPO TA cloning vector. The antisense primer sequences were 5′-ACAGTCTAGAGTTTTAACGCCAAGTTCGGTTTC-3′ and sense primer sequence was same as in full-length. The sequences of all the constructs were confirmed by sequencing. Both full-length and the HECT-deleted form of E6-AP were expressed as a fusion of V5 and His tags. The E6-AP knockdown constructs were made into pcDNA™6.2-GW/±EmGFP-miR vector using BLOCK-iT ™ Pol II miR RNAi Expression Vector Kit according to the manufacturer's instruction. The sequences of single-stranded oligonucleotides that were annealed and cloned were as follows: Top strand, TGCTGTTCAACTGCTGTCCTTGAA CTGTTTTGGCCACT GACTGACAGTTCAAGCAGCAGTTGAA; bot-

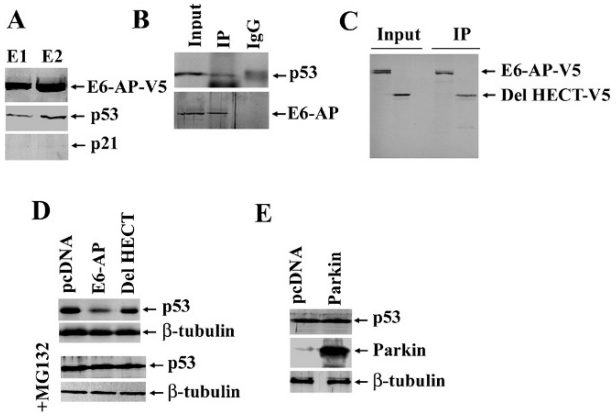

Figure 1. E6-AP interacts with p53 and decreases its levels in Cos-1 cells. (*A*) Cos-1 cells were transiently transfected with full-length E6-AP construct. At 24 h after post transfection, cells were lysed and processed for purification through Ni-NTA purification system as described in the Materials and methods. The eluted fractions (E1 and E2) were probed with V5, p53 and p21 antibodies. (*B*) Cos-1 cell lysates were co-immunoprecipitated with E6-AP antibody and blots were detected with p53 and E6-AP antibodies. (*C*) Cells were overexpressed with full-length and HECT domain-deleted constructs of E6-AP. After 24 h, cell lysates were made and subjected to co-immunoprecipitation using p53 antibody. Blot was detected with V5 antibody. (*D*) Cos-1 cells were transiently transfected with full-length and HECT-deleted constructs of E6-AP. At 48 h after post transfection, cells were collected and subjected to immunoblotting using p53 and β-tubulin antibodies. In some experiment MG132 (10 μM) was added for 12 h before collecting the cells. (*E*) Cells were transfected with a parkin construct in a similar way to that described in (*D*). Blots were detected with parkin, p53 and β-tubulin antibodies.

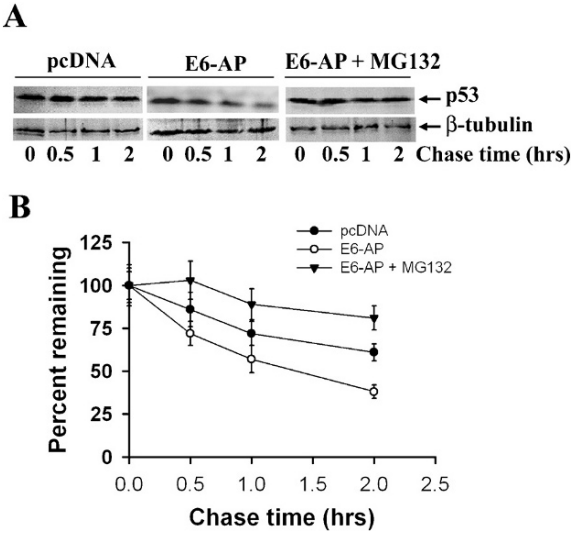

Figure 2. E6-AP increases the turnover of p53 in neuro 2a. (*A*) Neuro 2a cells were transiently transfected with either empty pcDNA or E6-AP plasmid. At 24 h post transfection, cells were treated with cycloheximide (15 μg/ml) and chased in the presence or absence of 10 μM MG132 for different periods. Blots were detected with p53 and β-tubulin antibodies. (*B*) p53 protein levels in the chase experiment described above were quantified for two independent experiments, each performed in duplicate. Quantitation was performed using NIH Image analysis software. Data were normalized against β-tubulin. E6-AP overexpression significantly decreased ($p<0.01$) the levels of p53 protein in comparison with control at all the time point tested.

tom strand, CAAGTTGACGACAGGAACTTGACAAAACC GGTGACTGACTGTCAAGT TCGTCGTCAACTTGTCC. The oligonucleotides were designed using Invitrogen's RNAi designer. The negative control plasmid (provided by Invitrogen) contains an insert that can form a hairpin structure that is processed into matured miRNA, but is predicted not to target any vertebrate gene. Both control and E6-AP-miRNA plasmids express GFP. Therefore, the control and E6-AP knockdown cells can be visualized under fluorescence microscope. The expression plasmid for p53 and parkin were kind gifts from Dr. N. Nukina and Dr. Y. Mizuno, respectively. Both p53 and parkin were expressed with V5 and His tags. The plasmid pp53-TA-Luc was purchased from BD Biosciences.

Cell culture, transfection and purification of recombinant proteins. Neuro 2a and Cos-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % heat-inactivated fetal bovine serum and antibiotics penicillin/streptomycin. For routine experiments, the cells were plated into six-well tissue culture plates at subconfluent density. After 24 h of plating, cells were transfected with various plasmids using Lipofactamine® 2000 according to the manufacturer's instruction and the cells were processed for immunoprecipitation, immunoblotting and immunofluorescence experiments. The transfection efficiency for Cos-1 and neuro 2a cells were about 80–90 % and 70–80 %, respectively.

For the purification of full-length and HECT domain-deleted E6-AP, p53 and parkin, the Cos-1 cells were transiently transfected with the respective plasmids for 48 h. The collected cells were then subjected to purification of 6×His-tagged recombinant proteins using Ni-NTA purification system according to the manufacturer's instruction.

TUNEL assay. Neuro 2a cells were plated into two-well chamber slides and on the following day cells were transiently transfected with control and E6-AP-miRNA plasmids. After 4 days of transfection, cells were washed twice with PBS, fixed with 4 % paraformaldehyde in PBS for 20 min, permeabilized with 0.5 % Triton X-100 in PBS for 5 min, washed extensively, and then blocked with 4 % BSA in 50 mM Tris, pH 7.5, 0.15 M NaCl, 0.05 % Tween (TBST) for 1 h. The cells were then subjected to TUNEL staining according to the manufacturer's instructions. The slides were mounted with Vetashield mounting media containing DAPI.

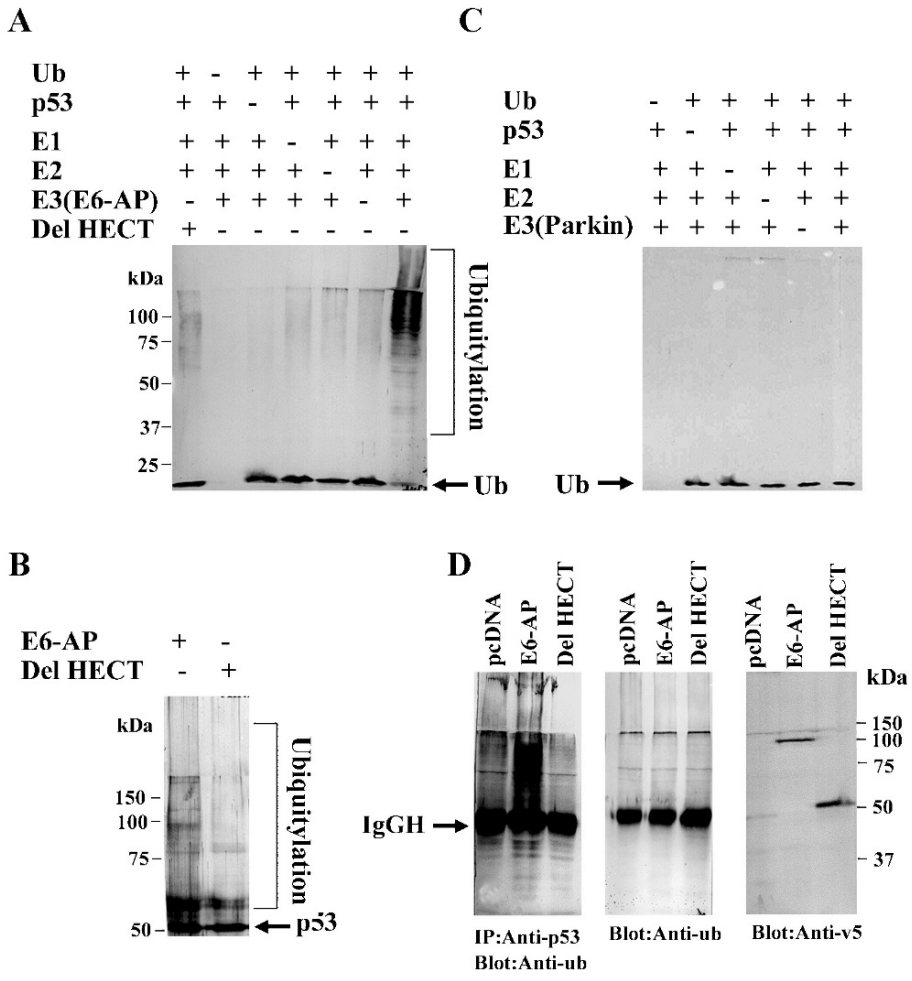

Figure 3. Ubiquitylation of p53 by E6-AP. (*A*, *B*) Affinity-purified p53 was incubated with purified E6-AP, HECT-deleted E6-AP along with ubiquitin (Ub), E1 and E2 as described in the Materials and methods. Blots were detected with ubiquitin (*A*) and p53 (*B*) antibodies. (*C*) Affinity-purified p53 was incubated with purified parkin in the similar way to that described in (*A*). Blots were detected with ubiquitin antibody. (*D*) Neuro 2a cells were transiently transfected with full-length and HECT domain-deleted constructs of E6-AP. After 48 h, cell lysates were made and processed for co-immunoprecipitation using p53 antibody. Blots were detected with ubiquitin (left and middle panel) and V5 (right panel) antibodies.

***In vitro* ubiquitylation assay.** The *in vitro* ubiquitylation assay was performed as described earlier [30]. Briefly, the purified p53 or parkin were incubated in a reaction volume of 50 µl 50 mM Tris-HCl, pH 7.4 containing 50 ng E1, 500 ng E2 (UBCH7), 2 µg of either E6-AP or HECT-deleted E6-AP, 6 µg bovine ubiquitin, 1 mM DTT, 2 mM $MgCl_2$ and 4 mM ATP. The incubation was carried out at 30°C for 2 h. The reaction was terminated by the addition of SDS-sample buffer, boiled and separated through 10% SDS-PAGE. Blots were probed with either ubiquitin or p53 antibody.

Co-immunoprecipitation and immunoblotting experiment. Neuro 2a and Cos-1 cells were transiently transfected with full-length and HECT domain-deleted E6-AP plasmids. After 24 h, cells were washed with cold PBS, scraped, pelleted by centrifugation, and lysed on ice for 30 min with NP-40 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, complete protease inhibitor cocktail). Cell lysates were briefly sonicated, centrifuged for 10 min at 15 000 *g* at 4°C and the supernatants (total soluble extract) were used for immunoprecipitation as described earlier [31, 32]. For each immunoprecipitation experiment, approximately 200 µg protein in 0.2 ml NP40 lysis buffer was incubated with 5 µl (2.5 µg) p53 antibody. After overnight incubation at 4°C with rotation, 20 µl protein G-agarose beads were added, and incubation was continued at 4°C for 5 h. The beads were washed six times with NP-40 lysis buffer. Bound proteins were eluted from the beads with SDS (1×) sample buffer, vortexed, boiled for 5 min, and analyzed by immunoblotting. The total cell lysate or the immunoprecipitated proteins were separated through SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were successively incubated in blocking buffer (5% skim milk in TBST), with primary antibody in TBST, and then with secondary antibody conjugated with alkaline phosphatase in TBST. Detection was carried out using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro -3-indox-

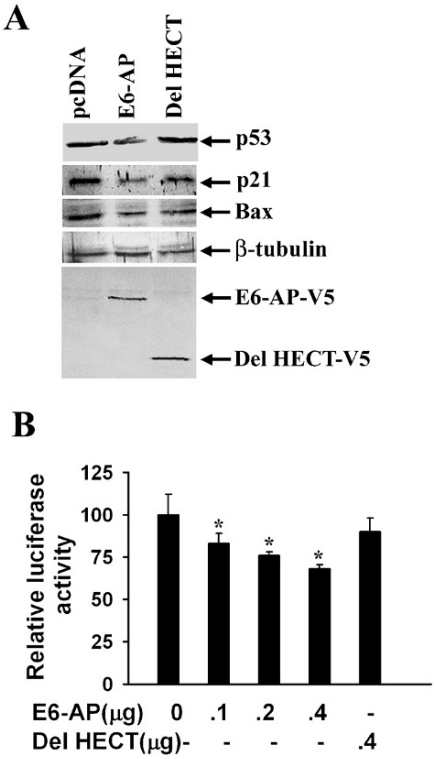

Figure 4. Overexpression of E6-AP decreases the level of p53 and p53-dependent transcription. (*A*) Neuro 2a cells were transiently transfected with full-length and HECT domain-deleted E6-AP plasmids. After 48 h, cells were collected and subjected to immunoblotting using p53, p21, bax and β-tubulin antibodies. (*B*) Cells were plated onto 24-well tissue culture plates, transfected with full-length and HECT domain-deleted E6-AP plasmids along with pp53-TA-Luc (a reporter plasmid expressing firefly luciferase under control of p53-response element). At 24 h post transfection, cell lysates were made and processed for luciferase enzyme assay as described in the Materials and methods. Values are means ± SD of three independent experiments each performed in triplicates. *$p<0.05$ as compared to control.

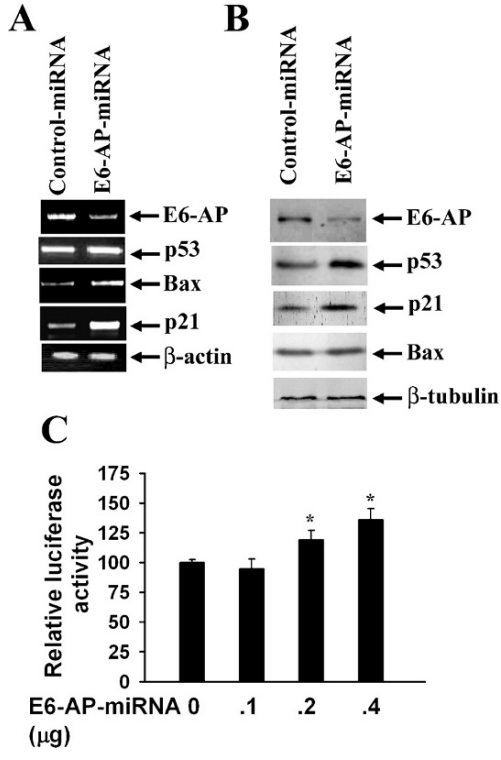

Figure 5. Partial knockdown of E6-AP increases the levels of p53 and p53-dependent gene transcription. Neuro 2a cells were transfected with control and E6-AP-miRNA constructs, and 48 h later the cells were collected and subjected to RNA extraction, followed by RT-PCR (*A*) and immunoblot (*B*) analysis. (*C*) Cells were transfected with E6-AP-miRNA plasmid along with pp53-TA-Luc. After 48 h, cells were collected and processed for luciferase assay. Values are means ± SD of three independent experiments each performed in triplicates. *$p<0.05$ as compared to control.

yl phosphate (BCIP). All primary antibodies were used in 1:1000 dilutions for immunoblotting except V5, which was used in 1:5000 dilutions.

Immunofluorescence techniques. Neuro 2a cells grown in chamber slides were transiently transfected with appropriate plasmids. At 48 h after transfection, cells were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 20 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min, washed extensively, and then blocked with 5% nonfat dried milk in TBST for 1 h. The cells were then incubated with appropriate primary antibody overnight at 4°C. After several washings with TBST, cells were incubated with appropriate fluorescent-labeled secondary antibody (1:500 dilutions) for 1 h, washed several times, and then mounted. Samples were observed using a fluorescence microscope, and digital image were assembled using Adobe Photoshop. The p53 antibody was used in 1:500 dilutions and V5 antibody was used in 1:2500 dilutions.

Degradation experiment. Neuro 2a cells were plated in a six-well tissue culture plate and, on the following day, cells were transiently transfected with empty pcDNA or E6-AP plasmids. After 24 h, cells were chased with 15 μg/ml cycloheximide for different time periods in the presence or absence of MG132. Cells collected at each time point were then processed for immunoblotting using antibodies against p53 and β-tubulin. Statistical analysis was performed using Student *t*-test and $p<0.05$ was considered to indicate statistical significance.

Reporter gene assay. Neuro 2a cells were plated into six-well tissue culture plates at a subconfluent density. After 24 h, cells were transiently transfected with either E6-AP full-length or E6-AP-miRNA plasmids along with pp53-TA-luciferase plasmids (a reporter plasmid expressing firefly luciferase under the control of p53 response element). At 24 h post

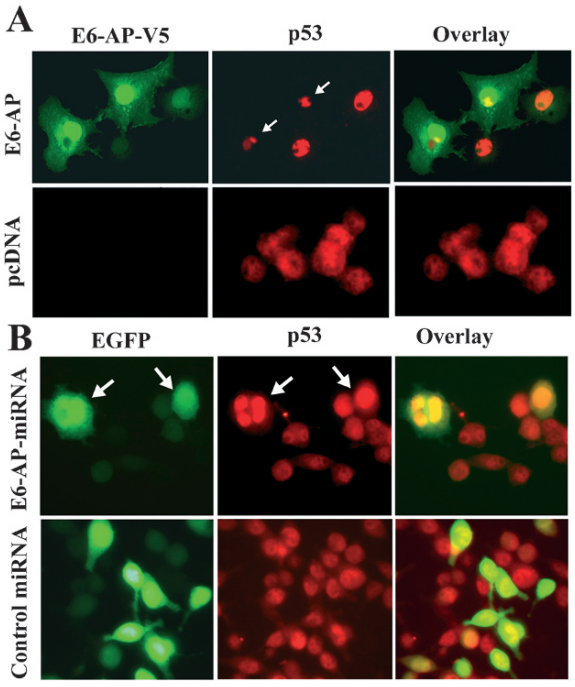

Figure 6. Over expression of E6-AP decreases, whereas partial knockdown increases, the cellular levels of p53. (*A*) Neuro 2a cells were plated onto two-well chamber slides and transfected with empty pcDNA or E6-AP plasmids. After 48 h, cells were processed for double immunofluorescence staining using V5 and p53 antibodies. FITC-conjugated secondary antibody was used to label the overexpressed E6-AP, while rhodamine-conjugated secondary antibody was used to label p53. Arrow indicates the E6-AP-overexpressing cells that have lower levels of p53. (*B*) Neuro 2a cells were transfected with control and E6-AP-miRNA plasmids as described in Figure 5. Cells were then processed for immunofluorescence staining of p53. The rhodamine-conjugated secondary antibody was used to label p53. Arrow indicates the E6-AP knockdown cells that show comparatively higher levels of p53.

transfection, cells were collected and then processed for luciferase assay according to the manufacturer's instructions. The luciferase activities were normalized with equal amounts of protein and expressed relative to p53-induced promoter activity. Statistical analysis was performed using Student *t*-test and $p<0.05$ was considered to indicate statistical significance.

RT-PCR analysis. The total RNA was extracted using TRIzol reagent and semi-quantitative RT-PCR was carried out with a RT-PCR kit (Invitrogen). The primer sequences for E6-AP, p53, bax and p21 were as follows: E6-APF, 5′-AACTGAGGGCTGTGGAAATG-3′; E6-APR, 5′-TCCGAAAGCTCAGAACCAGT-3′; p53F, 5′-GCAATGACTGCCATGGAGGAG-3′; p53R, 5′-CTTCTTGTAGATGGCCATGGC-3′; BaxF, 5′-GCCATGGACGGGTCC GGGGAG-3′; BaxR, 5′-GCCCATCTTCCAGAT-3′; p21F, 5′-GCCATGTCCAATCCT GGT-3′; p21R, 5′-GGGTTTTCTCTTGCAGAA-3′.

RNAi experiments. Neuro 2a cells were plated into six-well tissue culture plates and the following day the cells were transiently transfected with either E6-AP-miRNA or control-miRNA plasmids. After 24 h, cells were collected and processed for RT-PCR, immunoblotting and luciferase assays. To study the effect of E6-AP deficiency on cell growth, the neuro 2a cells were transfected with control and E6-AP-miRNA plasmids and the cell growth was monitored at 24, 48, 72 and 96 h of post transfection.

Results

E6-AP interacts with p53 and increases its turnover in Cos-1 and neuro 2a cells. The role of E6-AP in the degradation of p53 in HPV-negative cells is not very clear. Therefore, we investigated the possible role of E6-AP in the regulation of p53 levels in two different HPV-negative cells, Cos-1 and neuro 2a. First, we checked the interaction of E6-AP with p53 in the Cos-1 cells. Cells were overexpressed with E6-AP and the cell lysates were passed through the Ni-affinity column to purify the His-tagged E6-AP proteins. The p53 was co-eluted with the E6-AP, whereas p21 (a p53-inducible protein and a cell cycle regulatory protein) was completely absent in the eluted fraction (Fig. 1A). p53 was also co-eluted with the HECT domain-deleted form of E6-AP (data not shown). Next we performed co-immunoprecipitation experiments to check the possible interaction of E6-AP with the p53. In one experiment, the cell lysate was co-immunoprecipitated with E6-AP antibody and the blots were probed with E6-AP and p53 antibodies (Fig. 1B) and in a second experiment, the E6-AP overexpressed Cos-1 cell lysate was co-immunoprecipitated with p53 antibody and blot was probed with V5 antibody (Fig. 1C). In both co-immunoprecipitation experiments, endogenous and overexpressed E6-AP interacted with p53 (Fig. 1B, C). The HECT domain-deleted E6-AP was also co-immunoprecipitated with the p53 (Fig. 1C), suggesting that p53 possibly interacts with the N-terminal domain of E6-AP. The overexpression of E6-AP in the Cos-1 cells also decreased the steady state levels of p53, which could be prevented by addition of proteasome inhibitor MG132 (Fig. 1D). The E6-AP-induced degradation of p53 was abolished upon deletion of HECT domain of E6-AP. E6-AP also interacted with p53 and reduced the p53 levels in the neuro 2a cells. The overexpression of another ubiquitin ligase, parkin did not have any effect on the steady state levels of p53 (Fig. 1E). To further check the role of E6-AP on the turnover of cellular p53, we performed cycloheximide chase experiments. As shown in Figure 2, overexpression

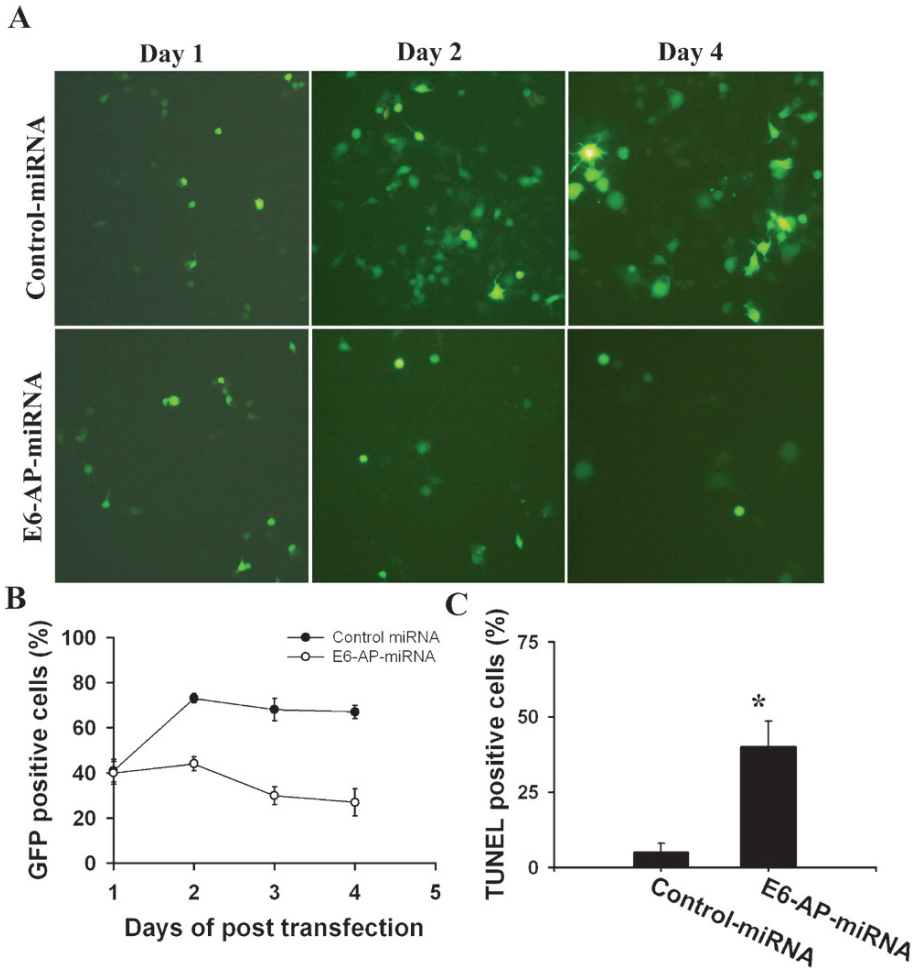

Figure 7. Down-regulation of E6-AP induces cell death. Neuro 2a cells were transiently transfected with control and E6-AP-miRNA plasmids as described in Figure 5 and cell growth was monitored at 24, 48, 72, 96 h post transfection. Fluorescent and phase-contrast images (of same areas) were taken in the different fields at different time points and the percentages of GFP-positive cells were calculated. (*A*) A representative fluorescence image at 24, 48 and 96 h post transfection. (*B*) Percentage of GFP-positive cells at different days post transfection. Values are means ± SD of three independent experiments. (*C*) Percentage of transfected cells that are TUNEL positive in the control and E6-AP-miRNA-transfected group at day 4. Values are means ± SD of three independent experiments. *$p<0.001$ as compared to control-miRNA-transfected group.

of E6-AP into the neuro 2a cells significantly decreased the half-life of p53, and the proteasome inhibitor MG132 was able to prevent the degradation of p53.

E6-AP promotes ubiquitylation of p53. Since E6-AP interacts with and increases the turnover of p53 in Cos-1 and neuro 2a cells, we next tested its role on the ubiquitylation of p53. In an *in vitro* ubiquitylation assay, the affinity-purified p53 was incubated with purified E6-AP along with E1, E2 (UBCH7) and ubiquitin. The blots were detected with either ubiquitin or p53 antibody. As shown in Figure 3A and B, E6-AP increased the ubiquitylation of p53, which could be prevented by removal of HECT domain. In a similar *in vitro* ubiquitylation assay, parkin was unable to ubiquitylate p53 (Fig. 3C). Next, we checked the role of E6-AP in the ubiquitylation of p53 in the neuro 2a cells. The cells were transiently transfected with full-length and HECT-deleted constructs of E6-AP; the cells were collected 48 h later, and lysates were made and processed for co-immunoprecipitation using anti-p53. The blots were detected with ubiquitin and V5 antibodies. As shown in Figure 3D, the overexpression of E6-AP into the neuro 2a cells also induced the ubiquitylation of p53, which was HECT domain dependent.

Overexpression of E6-AP decreases, whereas knockdown increases, the cellular levels of p53. We observed that the overexpression of E6-AP decreased the levels of p53 in the Cos-1 cells. We further tested the role of E6-AP on the turnover of p53 in the neuro 2a cells. The neuro 2a cells were overexpressed with full-length and HECT domain-deleted constructs of E6-AP for 48 h and the levels of p53 and two p53-dependent genes, p21 and bax were checked. As shown in Figure 4, the protein levels of p53, p21 and bax decreased upon overexpression of E6-AP. Overexpression of E6-AP also significantly attenuated the p53-dependent transcription (Fig. 4B). The decrease in the p53-dependent transcription was specific because under similar experimental conditions E6-AP activated glucocorticoid receptor-mediated transcription (data not shown). The removal of HECT domain of E6-AP abolished the degradation of p53 and reduction of p53-dependent

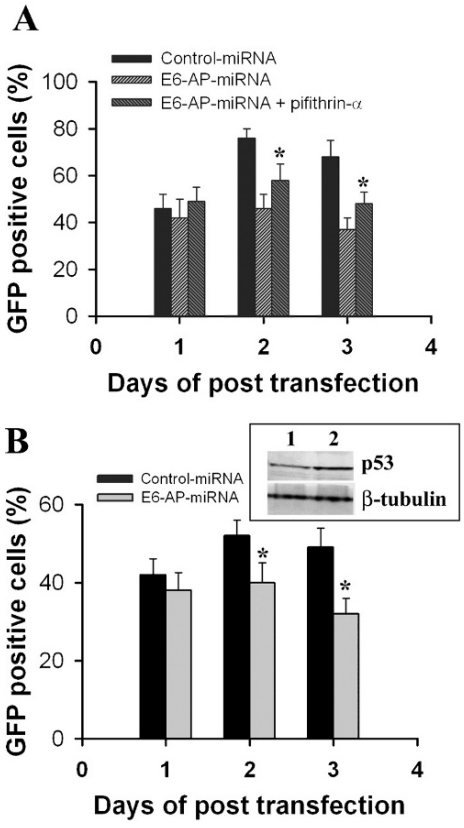

Figure 8. (*A*) Effect of pifithrin-α (a chemical inhibitor of p53 that reversibly blocks p53-dependent transcriptional activation) on the viability of E6-AP-deficient neuro 2a cells. Neuro 2a cells were transiently transfected with E6-AP-miRNA plasmids as described above. At 12 h after transfection, media was replaced, pifithrin-α (10 μM) was added and the growth of GFP-positive cells were monitored at different time periods. *$p<0.01$ as compared to E6-AP-miRNA-transfected group. (*B*) Effect of differentiation of the death of E6-AP-deficient neuro 2a cells. Neuro 2a cells were transfected with control and E6-AP-miRNA plasmids. After 12 h, the medium was replaced and 5mM dbcAMP was added; GFP-positive cells were counted at different time periods. *$p<0.01$ as compared to control-miRNA-transfected group. Inset shows the levels of p53 in the control (1) and E6-AP-miRNA (2)-transfected cells treated with dbcAMP for 2 days.

transcription. Since the overexpression of E6-AP induces the degradation p53, we further checked the effect of down-regulation of E6-AP on the degradation of p53 and p53-dependent transcription. Neuro 2a cells were transiently transfected with control and E6-AP-miRNA plasmids. After 48 h, cells were collected and mRNA and proteins levels of E6-AP, p53, p21 and bax were analyzed. Figure 5 shows that partial knockdown of E6-AP resulted an increase in the mRNA as well as protein levels of p53, p21 and bax. E6-AP knockdown caused about a 1.5-, 2- and 6-fold increase in the mRNA levels of p53, bax and p21, respectively. The protein levels of p53 was increased to about 2-fold in the E6-AP-deficient neuro 2a cells. The increase in the mRNA levels of p53 is rather surprising and suggests that E6-AP might also play an important role in the regulation of transcription of p53. Down-regulation of E6-AP also significantly increased the p53-dependent transcription (Fig. 5C). Transfection of control miRNA did not have any effect on p53-dependent transcription (data not shown).

We further confirmed the decreased levels of p53 in the E6-AP overexpressed neuro 2a cell by double immunofluorescence staining using V5 and p53 antibodies (Fig. 6, top panel). E6-AP was predominantly localized into the nucleus with diffuse cytoplasmic staining. The E6-AP overexpressed cells showed very low levels of nuclear p53. Similar results were also observed in Cos-1 cells. Next we checked the levels of p53 in the E6-AP-deficient cells. The neuro 2a cells were transiently transfected with control and E6-AP-miRNA plasmids for 48 h and then subjected to immunofluorescence staining using p53 antibody. As expected, the levels of p53 was higher in the E6-AP-deficient cells in comparison with non-transfected cells (Fig. 6, lower panel). E6-AP-deficient cells showed GFP fluorescence, as indicated by arrow. The control miRNA did not have any effect on cellular p53 levels. The control plasmid contains an insert that can form a hairpin structure that is processed into matured miRNA, but is predicted not to target any vertebrate gene.

Knockdown of E6-AP induces cell death. The increased levels of p53 in the E6-AP-deficient cells prompted us to investigate the impact of E6-AP knockdown on cell growth and survival. The neuro 2a cells were plated at a very low density and then transiently transfected with control and E6-AP-miRNA plasmids. The cell growth of the transfected cells (GFP-positive) were then monitored at different time points. As shown in Figure 7, the percentage of GFP-positive E6-AP-miRNA-transfected cells were significantly reduced compared to GFP-positive control-miRNA-transfected cells at different days tested. The percentage E6-AP-miRNA-transfected cells at day 1 was fairly similar to that of the control, but was reduced to about 40 % at days 3 and 4 in comparison with control (Fig. 7B). We also observed a significant increase in TUNEL-positive cells in the E6-AP-miRNA-transfected cells (Fig. 7C). Next we tested the role of p53 on the viability of E6-AP-deficient neuro 2a cell. The cells were transfected with E6-AP-miRNA construct and then treated with pifithrin-α (a chemical inhibitor of p53 that reversibly blocks p53-dependent transcriptional activation). We observed that the treatment with pifithrin-α partially protected E6-AP-deficient cells against death (Fig. 8A). Finally, we checked the effect of differentiation on the viability of E6-AP-deficient cells. We treated the

control and E6-AP-miRNA transfected cells with dbcAMP and the numbers of GFP-positive cells were counted at different time points. Partial knockdown of E6-AP also induced the death of differentiated neuro 2a cells (Fig. 8B); however, the rate of cell death was much slower than that of the dividing cells. Differentiated neuro 2a cells that are deficient in E6-AP also showed increased p53 levels. E6-AP-deficient neuro 2a cells that were treated with dbcAMP also showed higher levels of p53 compared to control (Fig. 8B inset).

Discussion

E6-AP degrades tumor suppressor p53 in the HPV-positive cells with the help of E6 oncoprotein of the HPV [12, 13]. This phenomenon could lead to the rapid growth and development of the cervical cancer cells. However, the role of E6-AP in the degradation p53 in the HPV-negative cells is confusing. A number of report demonstrated that E6-AP is unable to degrade p53 without E6 oncoprotein [24–26]. In contrast, E6-AP maternal-deficient mice generated by Jiang et al. [27] showed an increased cytoplasmic accumulation of p53 in the different parts of the brain, particularly, in the cerebellar Purkinje cells, and also in hippocampal CA1, CA2 and CA3 neurons. Recently, a high level of p53 and apoptosis was demonstrated in the prostate gland in these E6-AP maternal-deficient mice [28]. However, another mouse model of AS generated by Miura et al. [29] did not exhibit any sign of p53 accumulation in the Purkinje cells or other cells of mice brain. Here, we have demonstrated that E6-AP is indeed involved in the degradation of p53 in the neuronal cells without the help of E6 oncoprotein. Additionally, we have also found that E6-AP regulates the p53 gene transcription.

First, we have shown that E6-AP is able to interact with and ubiquitylate p53, and thereby increases its degradation by proteasome in the neuro 2a cells. E6-AP also ubiquitylates p53 in an *in vitro* ubiquitylation assay. Secondly, RNAi-mediated knockdown of E6-AP stabilizes the p53 level and increases the transcription of various p53-dependent genes. Partial knockdown of E6-AP also decreases cell proliferation and increases apoptosis. Finally, we have found that the partial knockdown of E6-AP increases the p53 mRNA level in the neuro 2a cells.

In the HPV-positive cells, E6-AP and E6 complex works as a functional E3 ubiquitin ligase for the ubiquitylation of p53 [12, 13]. However, E6-AP was found to ubiquitylate several cellular proteins in the absence of E6 [18–22]. The binding of E6 with E6-AP can dramatically enhance the rate of p53 ubiquitylation either by increasing the ligase activity of E6-AP or by increasing the association of E6-AP with p53. In fact, in HPV-positive cervical cancer cells there has been a report of a complete shift of p53 degradation from ubiquitin ligase Mdm2 to E6.E6-AP [33]. Therefore, any E6-independent degradation of p53 would be difficult to detect in these cells. However, it is not clear why E6-AP is unable to degrade p53 in some HPV-negative cells [26]. Some cell-specific factor(s) might be playing an important role for E6-AP-dependent ubiquitylation of p53.

Mdm2-mediated ubiquitylation and degradation of p53 plays a central role in maintaining low levels of p53 in normal cells [34, 35]. The functional interplay between Mdm2 and p53 is very complex. The interaction between Mdm2 and p53 creates an autoregulatory feedback loop in which p53 increases transcription of Mdm2, and Mdm2 promotes ubiquitylation and degradation of p53 [35]. Ubiquitin ligases other than Mdm2 have recently been demonstrated to promote the degradation of p53 in normal cells including Pirh2, COP1, p300, ARF-BP1 and CHIP [36–40]. Therefore, several ubiquitin ligases are involved in the degradation of p53 to maintain its low levels in the cells. E6-AP could be another ubiquitin ligase involved in the regulation of p53 levels.

Another interesting observation is the transcriptional up-regulation of p53 in the E6-AP-deficient neuronal cells. This finding strongly suggests that under normal circumstances, E6-AP might be trying to regulate the transcription of p53. The ubiquitin-mediated degradation of various transcription factors plays an important role in transcriptional regulation of many genes [41, 42]. E6-AP might be involved in ubiquitylation and degradation of some transcription factor(s) that are associated with the transcription of p53. E6-AP is known to function as a transcriptional coactivator of various steroid hormone receptors and this coactivator function might be indirectly linked with the transcriptional repression of p53 [23, 43]. Alternately, E6-AP might works as a corepressor. Exploring the role of E6-AP in the transcriptional regulation of p53 or other genes could be an exciting area of research. Our result clearly suggests that E6-AP not only promotes the proteasomal degradation but also regulate transcription of p53 and therefore add another level of complexity in the regulation of p53. Since the level of p53 in the cells is tightly regulated by several ubiquitin ligases, it is expected that in the E6-AP-deficient cells, other ubiquitin ligases would try to reduce the higher level of p53. Failure to control the increased p53 levels would results in cell cycle arrest and apoptosis [44, 45]. In fact, apoptotic cell death has been observed in the prostate gland of E6-AP

maternal-deficient mice. In addition to increased levels of p53, the prostate gland of these mice also showed increased levels of bax, p21 and active caspase-9 and -3 [28]. We have also observed inhibition of cell growth and increased apoptosis in the E6-AP-knockdown neuro 2a cells. These results suggest that E6-AP could be an important regulator of p53 or it might regulate the turnover of some other protein involved in cell cycle progression and apoptosis. In postmitotic neurons, higher levels of p53 also might cause apoptosis, while the chronic presence of sublethal concentrations might induce neuronal dysfunction [46]. The E6-AP maternal-deficient mice exhibit severe cognitive and motor abnormalities without any gross structural alterations and neuronal loss in the hippocampus and cerebellum [27]. However, E6-AP-deficient mice transgenically overexpressing the expanded polyglutamine ataxin-1 specifically in the Purkinje cells showed altered Purkinje cell morphology and accelerated SCA1 pathology [47]. This strongly suggests the Purkinje cells of the E6-AP-deficient mice are more vulnerable to stress and p53 might be involved in the accelerated pathology.

Altogether, our results suggest that E6-AP not only promotes ubiquitylation of p53 for targeting proteasomal degradation but also regulates neuronal cell growth, and its loss of function in AS might induce p53-dependent neuronal dysfunction or death.

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