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E6-AP Promotes Misfolded Polyglutamine Proteins for Proteasomal Degradation and Suppresses Polyglutamine Protein Aggregation and Toxicity*[S]

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The accumulation of intracellular protein deposits as inclusion bodies is the common pathological hallmark of most age-related neurodegenerative disorders including polyglutamine diseases. Appearance of aggregates of the misfolded mutant disease proteins suggest that cells are unable to efficiently degrade them, and failure of clearance leads to the severe disturbances of the cellular quality control system. Recently, the quality control ubiquitin ligase CHIP has been shown to suppress the polyglutamine protein aggregation and toxicity. Here we have identified another ubiquitin ligase, called E6-AP, which is able to promote the proteasomal degradation of misfolded polyglutamine proteins and suppress the polyglutamine protein aggregation and polyglutamine protein-induced cell death. E6-AP interacts with the soluble misfolded polyglutamine protein and associates with their aggregates in both cellular and transgenic mouse models. Partial knockdown of E6-AP enhances the rate of aggregate formation and cell death mediated by the polyglutamine protein. Finally, we have demonstrated the up-regulation of E6-AP in the expanded polyglutamine protein-expressing cells as well as cells exposed to proteasomal stress. These findings suggest that E6-AP is a critical mediator of the neuronal response to misfolded polyglutamine proteins and represents a potential therapeutic target in the polyglutamine diseases.

The polyglutamine diseases are a group of inherited neurodegenerative disorders caused by an aberrant expansion of CAG repeats in the causative genes. Nine disorders have been identified caused by such expansions including Huntington disease (HD),[3] spinal and bulbar muscular atrophy (SBMA), dentatorubral pallidoluysian atrophy, and several spinocerebellar ataxias (SCA1, SCA2, SCA3, SCA6, SCA7, and SCA17) (1–4). All polyglutamine diseases are progressive, autosomal dominantly inherited (except for X-linked recessive SBMA), typically striking at midlife and have an extended period of neuronal dysfunction followed by neuronal loss and eventually death (1–4). The number of CAG repeats shares an inverse relationship with the age of onset and severity of the disease and successive generations of affected families experience anticipation. The transgenic animal studies have supported a toxic gain-of-function mechanism that leads to neuronal dysfunction and death (1–4), although recent evidence indicates that loss of normal protein function might also have a role in the disease pathogenesis (5, 6). The disease pathogenesis is explained on the basis of polyglutamine protein conformation change and aggregation, aberrant interactions, and interference with gene transcription, activation of caspases and other apoptotic pathways, and mitochondrial dysfunction (3, 4, 7–11).

The formation of neuronal intranuclear inclusions (NIIs) or aggregates of the disease protein is the major cytopathological hallmark of all these disorders. It has been suggested that the polyglutamine tract expansion leads to formation of misfolded conformations of the target protein. When cells sense the misfolded polyglutamine protein, initially it tries to refold, and failure to refold leads next to its degradation by the ubiquitin proteasome system. The appearance of aggregates of the misfolded expanded polyglutamine proteins indicates that cells are unable to efficiently degrade them, which eventually overwhelms the cellular quality control system (12–14). The recruitment of molecular chaperones, ubiquitin proteasome system components, to the polyglutamine aggregates could be an adaptive response of cells to get rid of the abnormal protein deposits (15–18). In fact, overexpression of various chaperones has been shown to suppress polyglutamine protein aggregation and polyglutamine protein-induced cell death (15, 17, 19–21). On the other hand, proteasome inhibitors have been shown to increase the aggregation of the expanded polyglutamine protein, suggesting that expanded polyglutamine proteins are degraded by proteasome (15, 16, 18, 22). Furthermore, a condi-

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[3] The abbreviations used are: HD, Huntington disease; SBMA, spinal and bulbar muscular atrophy; SCA, spinocerebellar ataxia; tNhtt, truncated N-terminal huntingtin; UPS, ubiquitin proteasome system; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; dbcAMP, N^6,2′-*O*-dibutyryl-adenosine-3′,5′-cyclic monophosphate; NIIs, neuronal intranuclear inclusions; GFP, green fluorescent protein; HECT, homologous to E6-AP C terminus; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole.

tional mouse model study showed that turning off the expression of mutant huntingtin leads to a reduction of NIIs and reversal of the disease phenotype, implying that the aggregate formation and the neuronal dysfunction in the cells are reversible (23, 24). The question remains of why the aggregation-prone polyglutamine proteins are not eliminated efficiently by proteasomal degradation before they form the insoluble protein deposits. This inefficiency may result from the inability to degrade the mutant protein at a rate equal to or faster than that at which aggregates form.

E3 ubiquitin ligases, particularly the quality control E3 ligases, recently gained huge importance and are implicated in the biology of neurodegenerative disorders involving protein misfolding including polyglutamine diseases (25–35). The quality control ligase, CHIP has been demonstrated to suppress aggregation and cell death mediated by various misfolded mutant disease proteins (25–35) and to slow the disease progression in the transgenic mouse model of polyglutamine diseases (33, 34). In normal cells, more than 30% of proteins are not folded properly and are eliminated in the endoplasmic reticulum, through endoplasmic reticulum-associated degradation (36). Cells have an excellent quality control system to deal with the abnormally folded protein. Therefore, it is quite possible that several E3 ligases might be involved in the ubiquitylation and degradation of misfolded proteins. Identification of other quality control ligases and understanding their role in disease pathogenesis could open up a new attractive therapeutic target for polyglutamine and other related neurodegenerative disorders.

In the present investigation, we have explored the possible neuroprotective role of E6-AP in polyglutamine diseases. E6-AP is the founding member of the HECT (homologous to E6-AP C terminus) domain family of E3 ubiquitin ligases that provides specific recognition of substrate protein (37, 38). E6-AP was discovered based on its ability to promote the ubiquitin-mediated degradation of tumor suppressor p53 in association with the E6 oncoprotein of the human papilloma virus (39, 40). Loss of function of E6-AP is associated with Angelman mental retardation syndrome (41). Here we report that the E6-AP promotes the proteasome-mediated degradation of polyglutamine proteins and rescues polyglutamine protein-induced cell death.

EXPERIMENTAL PROCEDURES

Materials—MG132, cycloheximide, N^6,2′-*O*-dibutyryladenosine-3′,5′-cyclic monophosphate (dbcAMP), 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), TRIzol reagent, rabbit polyclonal anti-ubiquitin, mouse monoclonal, anti-β-tubulin, and all cell culture reagents were obtained from Sigma. Lipofectamine® 2000, optiMEM, ponasterone A, mouse monoclonal V5 antibody, RT-PCR kits, and Block-IT™ Pol II miRNA RNAi expression vector kit were purchased from Invitrogen. Protein G-agarose beads and mouse monoclonal green fluorescent protein (GFP) antibody were purchased from Roche Applied Science. Rabbit polyclonal anti-E6-AP, goat polyclonal anti-lamin B, and mouse monoclonal anti-Hsp70 were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-E6-AP was purchased from BD Pharmingen and rabbit polyclonal anti-HDJ-1 was from StressGen Biotechnologies. Goat anti-mouse IgG-FITC and IgG-rhodamine, AP-conjugated anti-mouse, and anti-rabbit IgG were purchased from Vector Laboratories.

Expression Plasmids and Stable Cell Lines—The truncated N-terminal huntingtin (tNhtt) expression constructs, pIND-tNhtt-EGFP-16Q, pIND-tNhtt-150Q, and the generation of stable cell lines of these constructs in an ecdysone inducible systems have been described previously (42). The construction of plasmids, pEGFP-N1-MJD(t)-20CAG, pEGFP-N1-MJD(t)-80CAG, pEGFP-N1-MJD(t)-130CAG, pEGFP-N1-MJD(f)-20CAG, and pEGFP-N1-MJD(f)-130CAG were described elsewhere (43). The full-length E6-AP cDNA was isolated from neuro2a total RNA using RT-PCR (TaKaRa Biomedicals) and then the PCR product was cloned into the pcDNA3.1 TOPO TA cloning vector. Primer sequences were: 5′-ACCATGGAG-CGAGCAGCTGCAAAG CATCT-3′ (sense) and 5′-GTACA-GCATGCCAAATCCTTTGGC-3′ (antisense). The HECT domain-deleted (amino acids 1–413) construct was made using PCR, subcloning the PCR product into the pcDNA3.1 TOPO vector. The antisense primer sequences were: 5′-ACAGTCTAGAG-TTTTAACGCCAAGTTCGGTTTC-3′ and the sense primer sequence was the same as full-length. The E6-AP knockdown construct was made into the pcDNA™6.2-GW/±EmGFP-miR vector using the BLOCK-iT™ PolII miR RNAi Expression Vector kit according to the manufacturer's instructions.

The oligonucleotides were designed using the Invitrogen RNAi designer. The negative control plasmid (provided by Invitrogen) contains an insert that can form a hairpin structure that is processed into mature miRNA, but is predicted not to target any vertebrate gene.

Cell Culture, Transfection, Cell Viability Assay, and Counting of Aggregates—The wild-type mouse neuro2a and COS-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and the antibiotics penicillin/streptomycin. The stable cell lines (HD 16Q and HD 150Q) were maintained in the same medium containing 0.4 mg/ml Zeocin and 0.4 mg/ml G418. One day prior to transfection, cells were plated into 6-well tissue culture plates at a subconfluent density. Cells were transiently transfected with expression vectors using the Lipofectamine® 2000 reagent according to the manufacturer's instructions. The transfection efficiency was about 80–90%. After 24 or 48 h of transfection, cells were used for immunofluorescence staining, co-immunoprecipitation, and immunoblotting. For the cell viability assay, cells were first transfected with different expression plasmids. 12 h later, cells were harvested and re-plated into 96-well plates (5×10^3 cells/well). Cells were then differentiated with 5 mM dbcAMP and induced with 1 μM ponasterone A for 3 days. The cell viability was measured by the MTT assay as described previously (42). Statistical analysis was performed using the Student's t test, and $p < 0.05$ indicated statistical significance. Aggregate formation was manually counted under a fluorescence microscope (~500 transfected cells in each case), and cells containing more than one aggregate were considered to have a single aggregate.

Co-immunoprecipitation and Immunoblotting Experiments—HD 16Q and HD 150Q cells were induced with ponasterone A for 24 h, and cells were washed with cold PBS, scraped, pelleted by centrifugation, and lysed on ice for 30 min with Nonidet P-40 lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, complete protease inhibitor mixture). In some experiments, neuro2a cells were transfected with either full-length or HECT domain-deleted constructs of E6-AP along with the truncated ataxin-3 construct containing 80 CAG repeats. 48 h later, cells were collected and processed as above. 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). For each immunoprecipitation experiment, 200 μg of protein in 0.2 ml of Nonidet P-40 lysis buffer was incubated either with 5 μl (2 μg) of GFP antibody or 4 μl (2 μg) of normal mouse IgG. 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 (50 mM Tris, pH 7.5, 0.15 M NaCl, 0.05% Tween)), with primary antibody in TBST, and then with secondary antibody conjugated with alkaline phosphatase in TBST. Detection was carried out using nitroblue tetrazolium and 5-bromo-4-chloro-3-indoxyl phosphate. All primary antibodies were used in 1:1000 dilutions for immunoblotting except V5, which was used in a 1:5000 dilution.

Immunofluorescence Techniques—HD 150Q cells grown in chamber slides were induced with ponasterone A for 48 h. In some experiments, neuro2a cells were transiently transfected with truncated ataxin-3 constructs along with E6-AP for 48 h. Cells were then 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, then blocked with 5% nonfat dried milk in TBST for 1 h. The primary antibody (mouse monoclonal anti-E6-AP, 1:250 dilution and anti-V5, 1:1000 dilution) incubation was carried out overnight at 4 °C. After several washing steps with TBST, cells were incubated with rhodamine-conjugated secondary antibody (1:500 dilutions) for 1 h, washed several times, and mounted. Samples were observed using a fluorescence microscope, and digital images were assembled using Adobe Photoshop. For immunofluorescence staining of E6-AP and ubiquitin in the control and transgenic mouse brain sections, the paraffin-embedded brain sections were deparaffinized, subjected to antigen retrieval, and then processed for staining as described earlier (18). The appropriate FITC- and rhodamine-conjugated secondary antibody was used to visualize expression and localization.

Degradation Assay—Neuro2a cells were plated in 6-well tissue culture plates, and on the following day, cells were transiently transfected with full-length ataxin-3 constructs containing 20 and 130 CAG with or without E6-AP. After 24 h of post-transfection, cells were chased with 15 μg/ml of cycloheximide for different time periods. Cells collected at each time point were then processed for immunoblotting by anti-GFP.

RT-PCR Analysis—Total RNA was extracted using the TRIzol reagent, and RT-PCR was carried out with an RT-PCR kit (Invitrogen). The primer sequences for E6-AP and β-actin were as follows: E6-APF, 5′-AACTGAGGGCTGTGGAAATG-3′; E6-APR, 5′-TCCGAAAGCTCAGAACCAGT-3′; ActinF, 5′-TACAGCTTCACCACC-3′; ActinR, 5′-ATGCCACAGGATTTC-3′. PCR conditions for E6-AP and β-actin were the same: an initial denaturation step at 94 °C for 4 min and then cycling through 94 °C for 30 s denaturation, 60 °C for 30 s annealing, 72 °C for 45 s extension, and a final extension step at 72 °C for 5 min. The cycle number for E6-AP was 32 and for β-actin was 23.

RNAi Experiments—HD 150Q cells were plated into 6-well tissue culture plates, and on the following day, cells were transiently transfected with either E6-AP miRNA or control miRNA plasmids. 24 h later, cells were induced with ponasterone A for different time periods, and aggregate formation was monitored under a fluorescence microscope. For the cell viability assay, transfected HD 16Q and HD 150Q cells were induced with ponasterone A and differentiated with dbcAMP for 3 days. The cell viability was monitored using the MTT assay.

RESULTS

The mRNA Level of E6-AP Is Dramatically Increased in the Expanded Polyglutamine Protein-expressing Cells—An earlier study had shown that SCA1 transgenic mice, deficient in E6-AP, demonstrated accelerated SCA1 pathology and reduced aggregate formation (22). But how the loss of function of E6-AP modulates the ataxin-1 aggregation and SCA1 pathology is not known as yet. Therefore, we began to explore the possible role of E6-AP on polyglutamine disease pathogenesis using cellular and transgenic mouse models of HD. Because E6-AP is an ubiquitin ligase, we hypothesized that it might be involved in the proteasome-mediated degradation of polyglutamine proteins. During the course of our work, we unexpectedly noticed that the expression of E6-AP was dramatically increased in expanded polyglutamine protein-expressing cells as well as cells treated with proteasome inhibitor. HD16Q and HD 150Q cells (the stable neuro2a cell lines in an inducible system that express tNhtt with normal (16Q) and expanded polyglutamine (150Q) proteins) were induced for 48 h, and total RNA was extracted. RT-PCR was performed and analyzed by agarose gel electrophoresis to detect the PCR product. mRNA levels of E6-AP were found to be significantly up-regulated in HD150Q cells in comparison with HD 16Q cells (Fig. 1, *A* and *B*). Approximately, a 2.5-fold increase in the levels of E6-AP mRNA was observed in HD 150Q cells in comparison with HD 16Q cells. Fig. 1*C* demonstrates the expression of truncated huntingtin with 16Q and 150Q. About 50–60% HD 150Q cells form aggregates upon 48 h of induction with 1 μM ponasterone A. The treatment of the proteasome inhibitor MG132 for 6 h with HD 16Q cells also dramatically induced E6-AP mRNA levels. We further confirmed the effect of expanded polyglutamine protein expression on E6-AP mRNA levels by transiently transfecting normal and polyglutamine-expanded ataxin-3 constructs into mouse neuro2a cells. After 48 h, total RNA was isolated, and RT-PCR was performed. E6-AP mRNA was also found to be significantly up-regulated (about 2-fold) in mutant ataxin-3-expressing cells (Fig. 1, *D* and *E*). Fig. 1*F* showed expression levels of truncated ataxin-3 with 20Q and

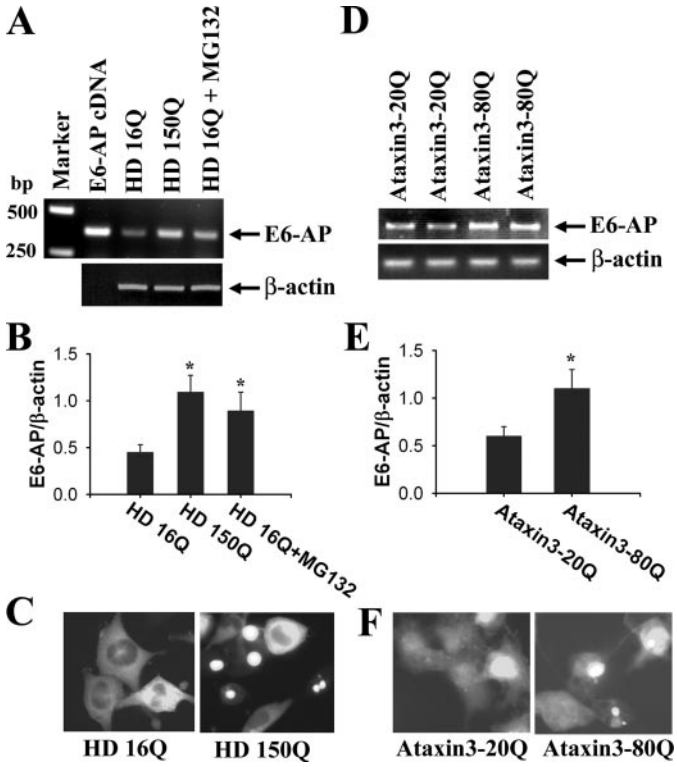

FIGURE 1. **Up-regulation of E6-AP mRNA levels in the expanded polyglutamine protein-expressing cells as well as cells treated with proteasome inhibitor.** *A*, HD 16Q and HD 150Q cells were plated into 6-well tissue culture plates and induced with 1 μM ponasterone A for 48 h. Cells were then processed for RNA extraction followed by RT-PCR analysis of E6-AP and β-actin. In some experiments, induced HD 16Q cells were incubated with 10 μM MG132 for 6 h, and then cells were processed for RT-PCR analysis as above. E6-AP cDNA was used as a positive control. *B*, quantitation of the E6-AP band intensities as shown in *A* collected from three independent experiments using the NIH image analysis software. Data were normalized using β-actin. Values are means ± S.D. *, $p < 0.01$ compared with HD 16Q cells. *C*, expression of truncated N-terminal huntingtin with 16Q and 150Q after 48 h of induction. *D*, mouse neuro2a cells were transiently transfected with normal and polyglutamine-expanded truncated ataxin-3. 48 h after transfection, total RNA was isolated, and RT-PCR was performed. *E*, quantitation of band intensities as shown in *D* collected from three independent experiments using the NIH image analysis software. Values are means ± S.D. *, $p < 0.01$ compared with ataxin-3 with 20Q. *F*, expression of truncated ataxin-3 with 20Q and 80Q after 48 h of transfection.

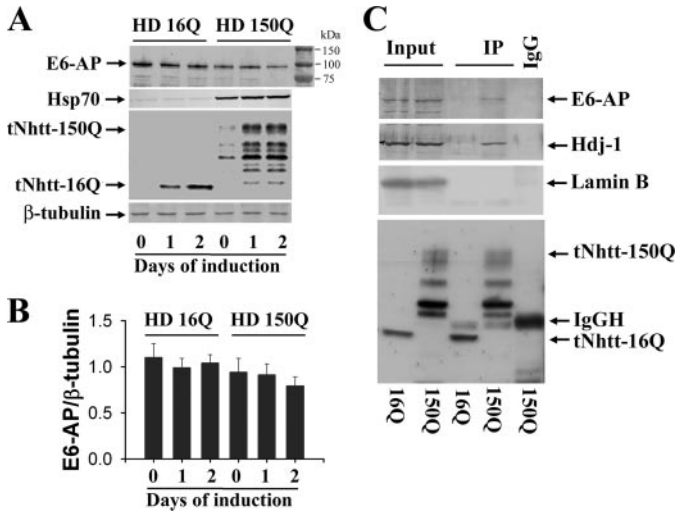

FIGURE 2. *A* and *B*, analysis of the protein level of E6-AP in normal and expanded polyglutamine protein-expressing cells. *A*, HD 16Q and HD 150Q cells were plated into 6-well tissue culture plates. On the following day, cells were left untreated or induced with 1 μM ponasterone A for different time periods as indicated in the figure. Cells collected at each time point were then processed for immunoblot analysis using E6-AP, Hsp70, GFP, and β-tubulin antibodies. *B*, quantitation of band intensities of the E6-AP blots collected from three independent experiments was performed using the NIH Image analysis software. Data were normalized using β-tubulin. Values are mean ± S.D. *C*, interaction of E6-AP with soluble expanded polyglutamine proteins. HD 16Q and HD 150Q cells were plated in a similar manner as described in *A*. 24 h after induction, cells were collected and processed for immunoprecipitation (*IP*) by anti-GFP. Blots were probed with antibodies against E6-AP, Hdj-1, Lamin B, and GFP (*lower blot*).

80Q after 48 h of transfection. Approximately, 40–50% of the transfected cells form aggregates during this period.

E6-AP Interacts with the Soluble Misfolded Expanded Polyglutamine Proteins and Associates with Their Aggregates—Next, we analyzed the protein levels of E6-AP in normal and expanded polyglutamine protein-expressing cells. HD 16Q and HD 150Q cells were induced for different days, and then the cell lysates were subjected to immunoblot analysis using E6-AP, Hsp70, GFP, and β-tubulin antibodies. To our surprise, we did not find any correlation of the protein levels of E6-AP with its mRNA levels in induced HD 150Q cells. Protein levels of E6-AP in uninduced HD 16Q and HD 150Q cells were almost similar and were slightly decreased in HD 150Q cells when induced for 2 days (Fig. 2, *A* and *B*). Under similar experimental conditions, HD 150Q cells showed very high levels of expression of Hsp70 (Fig. 2*A*). Levels of expression of Hsp70 in the uninduced HD 150Q cells were also very high. This is most likely due to the low levels of expression and aggregation of tNhtt-150Q protein without any induction (18). Similar results were also observed when we transiently transfected normal and polyglutamine expanded truncated ataxin-3 into neuro2a cells for 2 days (data not shown). Because there is no correlation of the protein and mRNA levels of E6-AP in the expanded polyglutamine protein-expressing cells, we thought that the decrease in the protein levels of E6-AP in the expanded polyglutamine protein-expressing cells could be due to its association with polyglutamine aggregates. To confirm this hypothesis, we first checked the interaction of E6-AP with soluble polyglutamine proteins. We have observed that E6-AP interacted with soluble expanded polyglutamine proteins (Fig. 2*C*). We used the Hdj-1 chaperone as a positive control that strongly interacted with the expanded polyglutamine protein (Fig. 2*C*). Next we tested the association of E6-AP with polyglutamine aggregates. HD 16Q and HD 150Q cells were induced for 2 days and then subjected to immunofluorescence staining of E6-AP. The E6-AP was localized in both the cytoplasm and nucleus with predominant nuclear staining in wild-type neuro2a cells (data not shown). The expression of tNhtt-16Q in HD 16Q cells did not alter the distribution profile of E6-AP in comparison with wild-type neuro2a cells (Fig. 3). However, we noticed that the expression of the tNhtt-150Q protein in HD 150Q cells caused recruitment of E6-AP to mutant huntingtin aggregates. We also observed reduction of the nuclear and cytoplasmic staining of E6-AP in these cells. We further checked the similar redistribution of E6-AP in the ataxin-3 aggregates. E6-AP was co-transfected along with truncated ataxin-3 constructs, and after 2 days of transfection, cells were processed for immunofluorescence experiments. The subcellular localization of ataxin-3 (contain-

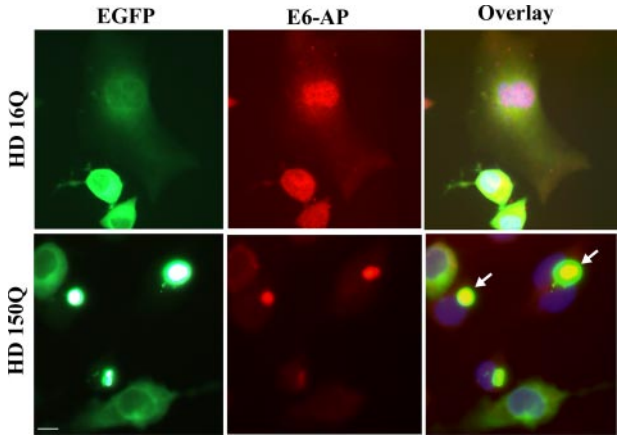

FIGURE 3. **Association of E6-AP with the polyglutamine aggregates.** The HD 16Q and HD 150Q cells were plated into 2-well chamber slides. Cells were induced with 1 μM ponasterone A for 48 h and processed for immunofluorescence staining using E6-AP antibody. Rhodamine-conjugated secondary antibody was used to stain the E6-AP. Nuclei were stained with DAPI. The *arrow* indicates the recruitment of E6-AP to the mutant huntingtin aggregates. Scale bar, 20 μm.

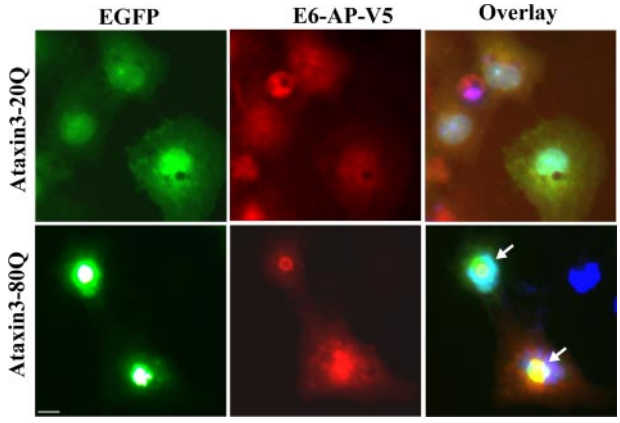

FIGURE 4. **Recruitment of E6-AP to ataxin-3 aggregates.** Neuro2a cells were plated into 2-well chamber slides, and on the following day, cells were transiently transfected with truncated ataxin-3-EGFP fusion constructs containing 20 CAG and 80 CAG along with E6-AP plasmid. 48 h after transfection, cells were subjected to immunofluorescence staining using V5 antibody. Rhodamine-conjugated secondary antibody was used to stain the overexpressed E6-AP-V5 protein. Nuclei were stained with DAPI. The *arrow* indicates the recruitment of E6-AP to ataxin-3 aggregates. Scale bar, 20 μm.

ing normal and expanded polyglutamine repeats) and E6-AP was very similar. They were predominantly localized in the nucleus with diffuse cytoplasmic distribution (Fig. 4). Overexpressed E6-AP was also recruited to ataxin-3 aggregates (Fig. 4). To further confirm the association of E6-AP with expanded polyglutamine proteins or their aggregates and exclude any interference by GFP, we transiently transfected truncated ataxin-3 constructs without the GFP tag. After 48 h of post-transfection, cells were processed for co-immunoprecipitation experiments using ataxin-3 antibody, and blots were detected with E6-AP and ataxin-3 antibodies. Cells were also subjected to double immunofluorescence staining using E6-AP and ataxin-3 antibodies to study co-localization. As expected, E6-AP interacted with the soluble polyglutamine expanded ataxin-3 and co-localized with ataxin-3 aggregates (data not shown). We have also found association of the HECT-deleted domain of E6-AP with polyglutamine aggregates (supplemental Fig. S1). These results suggest that the N-terminal domain of E6-AP most likely interacts with polyglutamine proteins.

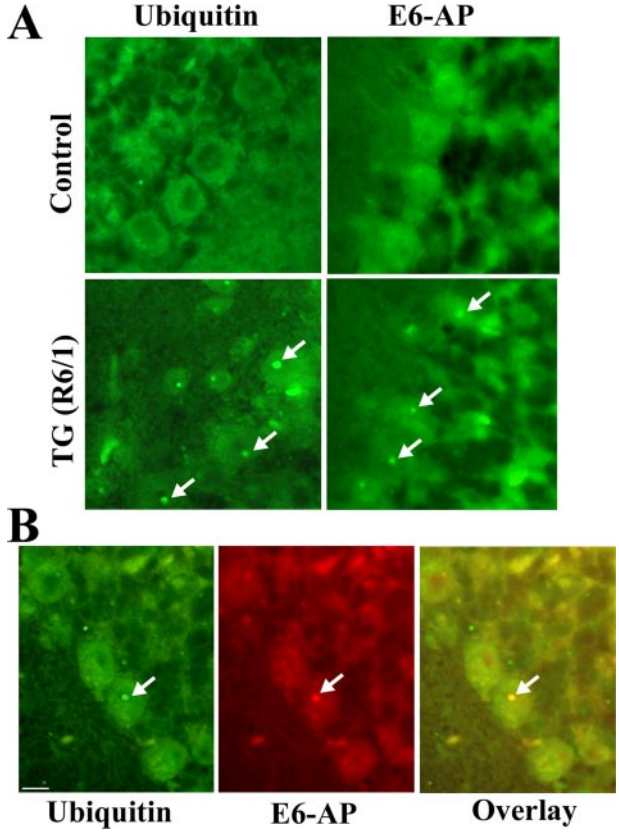

FIGURE 5. **Co-localization of E6-AP to the NIIs in the brain of HD exon1 (R6/1) transgenic mice.** *A*, immunofluorescence staining of ubiquitin and E6-AP in the cerebellum of control and R6/1 transgenic mice. Brain sections from about 36 week-old mice (both control and transgenic) were used. The FITC-conjugated anti-rabbit IgG was used to label ubiquitin, and FITC-conjugated anti-mouse IgG was used to label E6-AP. The *arrow* indicates the NIIs that were stained with ubiquitin and E6-AP. *B*, immunofluorescence double labeling of ubiquitin and E6-AP in the NIIs. The brain section was incubated with primary antibodies against E6-AP (anti-mouse) and ubiquitin (anti-rabbit). FITC-conjugated secondary antibody was used to label the ubiquitin, and rhodamine-conjugated secondary antibody was used to stain E6-AP. The *arrow* shows the NII that is co-localized with ubiquitin and E6-AP. Scale bar, 20 μm.

E6-AP Recruits to the NIIs in the Brain of HD Exon 1 Transgenic Mice—Because the E6-AP interacts and associates with polyglutamine aggregates in cellular models, we further tested its recruitment to the NIIs in the HD transgenic mouse brain. In the control mouse brain, the E6-AP was distributed primarily in the cerebellum, hippocampus, and cerebral cortex. The striatum showed very low levels of expression. E6-AP was also localized mostly in the nucleus with diffuse cytoplasmic staining in Purkinje cells, hippocampal, and cortical neurons. In the transgenic mouse brain, we observed clear recruitment of E6-AP to the NIIs particularly in the cerebellum, cerebral cortex, and hippocampus (Fig. 5 and supplemental Fig. S2). The double immunofluorescence staining further confirmed that the E6-AP colocalized with ubiquitin-positive aggregates (Fig. 5*B*). About 80–90% of ubiquitin-positive NIIs in Purkinje cells were E6-AP-positive, and about 30–40% of ubiquitin-positive NIIs in the cortex and hippocampus were E6-AP-positive. The redistribution of E6-AP to the NIIs often caused a decrease in E6-AP staining in the nucleus and cytoplasm.

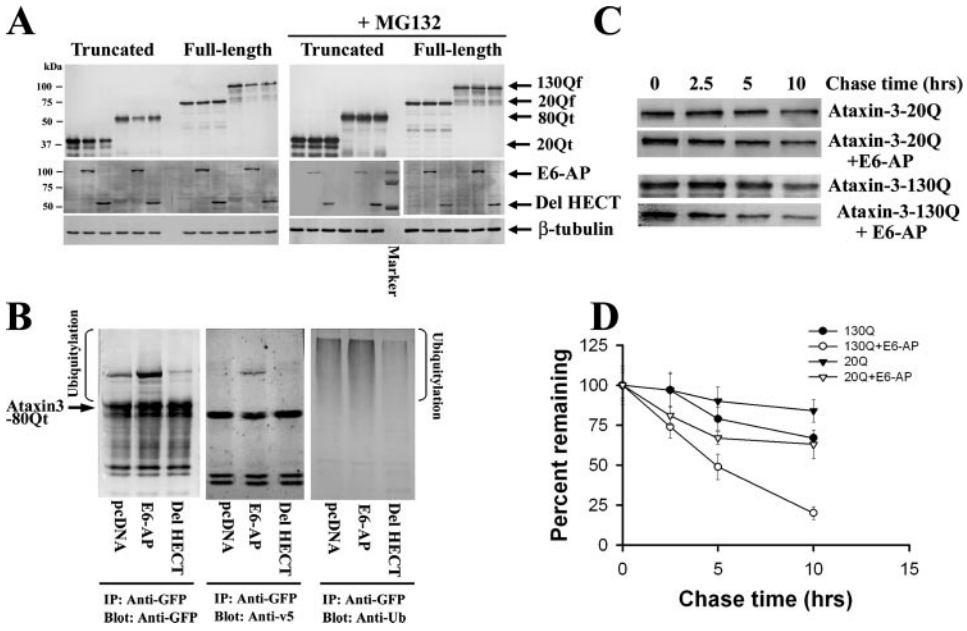

FIGURE 6. **E6-AP promotes ubiquitylation and degradation of expanded polyglutamine proteins.** *A*, neuro2a cells were transiently transfected with full-length (*f*) and truncated (*t*) ataxin-3 constructs containing normal and expanded CAG repeats along with either full-length or HECT-deleted constructs of E6-AP. 48 h after transfection, cells were collected and subjected to immunoblotting using GFP, V5, and β-tubulin antibodies. In some experiments, cells were treated with MG132 (10 μM for 8 h) before collecting (*right panel*). *B*, neuro2a cells were transiently transfected with the truncated ataxin-3 construct containing 80 CAG repeats along with either full-length or the HECT-deleted construct of E6-AP as above. Cells were then processed for co-immunoprecipitation using the GFP antibody as described under "Experimental Procedures." Blots were probed with antibodies against GFP, V5, and ubiquitin. *C*, neuro2a cells were transiently transfected with full-length ataxin-3 constructs (20 CAG and 130 CAG) either alone or along with E6-AP. 24 h later, cells were chased in the presence of 15 μg/ml of cycloheximide for different time periods as indicated in the figure. Cells were then collected and processed for immunoblot analysis using the GFP antibody. *D*, quantitation of band intensities of blots collected from three independent experiments was performed using the NIH Image analysis software. Values are mean ± S.D.

E6-AP Promotes Ubiquitylation and Degradation of Soluble Expanded Polyglutamine Proteins—We next tried to explore the functional significance of the interaction of E6-AP with polyglutamine proteins. Because the E6-AP is an ubiquitin ligase, we first tested its role on the ubiquitylation and degradation of polyglutamine proteins. The full-length and HECT domain-deleted constructs of E6-AP were co-transfected along with full-length or truncated ataxin-3 constructs containing normal and expanded CAG repeats to neuro2a cells. Cell lysates were then processed for immunoblotting using GFP, V5, and β-tubulin antibodies. Fig. 6*A* showed that the overexpression of E6-AP caused a significant decrease in levels of expanded polyglutamine proteins (both full-length and truncated), which can be prevented upon deletion of the HECT domain. Treatment with proteasome inhibitor MG132 also blocked the E6-AP-induced decrease in levels of expanded polyglutamine proteins, suggesting that E6-AP promotes the degradation of expanded polyglutamine proteins by the proteasome. Overexpression of E6-AP did not have any effect on levels of normal polyglutamine repeat length proteins. To study the role of E6-AP on the ubiquitylation of expanded polyglutamine proteins, we co-immunoprecipitated the ataxin-3–80Q protein with GFP antibody, and then the blots were sequentially probed with antibodies against GFP, V5, and ubiquitin. The overexpression of E6-AP caused increased accumulation of ubiquitylated derivatives of ataxin-3–80Q proteins (Fig. 6*B*, *left* and *right panels*), which can be prevented upon deletion of the HECT domain. Overexpressed E6-AP also interacted with soluble expanded polyglutamine proteins (Fig. 6*B*, *middle panel*).

Next, we studied the rate of degradation of expanded polyglutamine proteins upon overexpression of E6-AP. For these experiments, we used full-length ataxin-3 constructs containing 20 and 130 CAG, because full-length ataxin-3 with 130Q forms very few aggregates after 48 h of transfection (about 5–10%). Neuro2a cells were transiently transfected with ataxin-3 constructs either alone or along with E6-AP. 24 h later, cells were chased with cycloheximide. As shown in Fig. 6, *C* and *D*, full-length ataxin-3 with 20Q is degraded at a much slower rate compared with full-length ataxin-3 with 130Q. Overexpression of E6-AP dramatically increased the rate of degradation of full-length ataxin-3 containing 130Q. E6-AP also slowly enhanced the degradation of normal ataxin-3, most likely because of their misfolding as a result of overexpression.

Overexpression of E6-AP Suppresses Polyglutamine Protein Aggregation and Polyglutamine Protein-induced Cell Death—Because E6-AP promotes the degradation of polyglutamine proteins, we further tested its role on the suppression of polyglutamine protein aggregation and polyglutamine protein-induced cell death. HD 150Q cells were transiently transfected with varying concentrations of E6-AP; 24 h later, cells were induced, and aggregate formation was monitored under a fluorescence microscope. The overexpression of E6-AP significantly reduced polyglutamine protein aggregation, which was concentration-dependent (Fig. 7*A*). E6-AP overexpression also decreased the high molecular weight expanded polyglutamine protein complex retained in the stacking gel (Fig. 7*B*). Overexpression of the HECT domain-deleted construct of E6-AP had no effect on the rate of aggregate formation. Interestingly, we have found that the suppressive effect of aggregate formation of E6-AP was more pronounced when E6-AP was overexpressed along with Hsp70 (Fig. 7*C*). Overexpression of E6-AP also protected polyglutamine protein-induced cell death, and again, the protective effect was more prominent when E6-AP was co-expressed along with Hsp70 (Fig. 7*D*).

Partial Knockdown of E6-AP Enhances Polyglutamine Protein Aggregation and Polyglutamine Protein-induced Cell Death—Next, we checked the effect of partial knockdown of E6-AP on the rate of aggregate formation and cell death mediated by expanded polyglutamine proteins. We have developed a

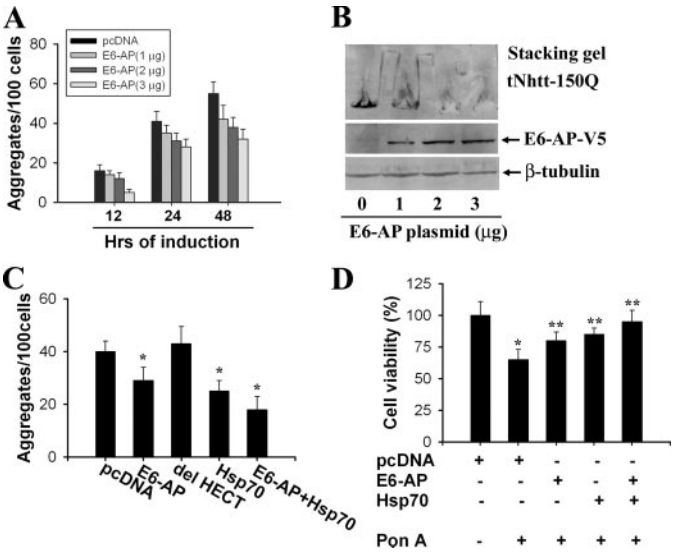

FIGURE 7. **Overexpression of E6-AP suppresses the aggregation and cell death mediated by expanded polyglutamine proteins.** *A*, HD150Q cells were transiently transfected with different concentrations of full-length E6-AP plasmid. 24 h later, cells were induced with 1 μM ponasterone A, and the aggregate formation was monitored under a fluorescence microscope for different time periods as indicated in the figure. *B*, HD 150Q cells were transfected with E6-AP and induced as above. Cells were then collected after 24 h of induction and subjected to immunoblot analysis using GFP, V5, and β-tubulin antibodies. The GFP antibody was used to detect the aggregated tNhtt-150Q protein in the stacking gel. *C*, HD 150Q cells were transiently transfected with full-length and HECT-deleted constructs of E6-AP either alone or along with Hsp70 (each 2 μg/well of a 6-well tissue culture plate). 24-h post-transfection, cells were induced with ponasterone A, and the aggregate formation was monitored 24 h after induction. *, $p < 0.01$ as compared with the pcDNA-transfected experiment. *D*, HD 150Q cells were transiently transfected with E6-AP independently or together with Hsp70 (each 2 μg/well of a 6-well tissue culture plate). 12 h later, cells were harvested and re-plated in a 96-well tissue culture plate. Cells were then differentiated with 5 mM dbcAMP and induced with 1 μM ponasterone A for 3 days. The cell viability was measured by MTT assay. Values are means ± S.D. of two independent experiments, each performed in triplicate. *, $p < 0.01$ compared with the uninduced and pcDNA-transfected groups. **, $p < 0.01$ compared with the pcDNA-transfected and ponasterone A-treated experiments.

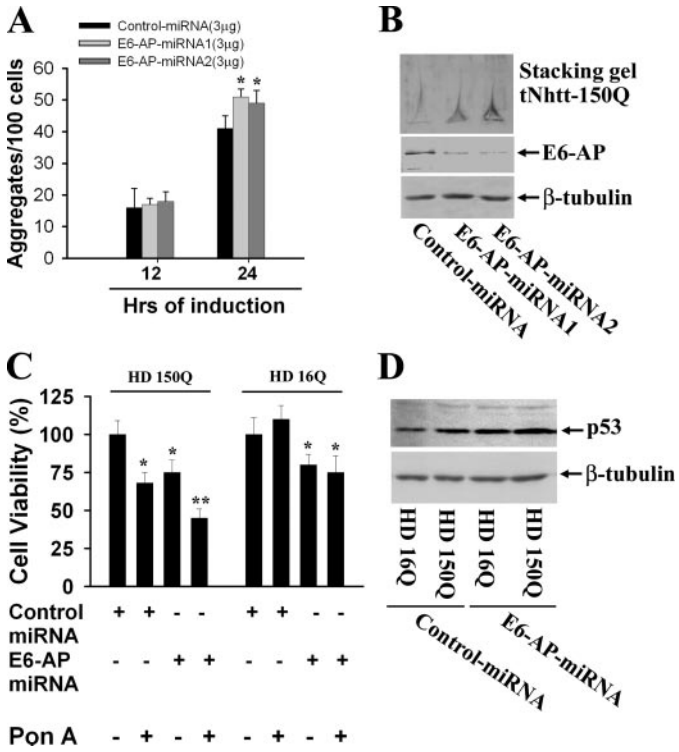

FIGURE 8. **Down-regulation of E6-AP promotes polyglutamine protein aggregation and polyglutamine protein-induced cell death.** *A* and *B*, HD 150Q cells were transfected with control and E6-AP miRNA plasmids (each 3 μg/well of a 6-well tissue culture plate) for 24 h, and then cells were induced with 1 μM ponasterone A. Aggregate formation was monitored for different time periods as indicated in the figure (*A*). Cells were then collected (after 48 h of transfection and 24 h of induction) and subjected to immunoblot analysis using GFP, E6-AP, and β-tubulin antibodies (*B*). The GFP antibody was used to detect the aggregated tNhtt-150Q protein in the stacking gel. *C*, HD 16Q and HD 150Q cells were transiently transfected with control and E6-AP miRNA1 plasmids (each 2 μg/well of a 6-well tissue culture plate). 12 h later, cells were harvested and re-plated in a 96-well tissue culture plate. Cells were then differentiated with 5 mM dbcAMP and induced with 1 μM ponasterone A for 3 days. The cell viability was measured by the MTT assay. Values are means ± S.D. of two independent experiments, each performed in triplicate. *, $p < 0.01$ compared with uninduced control miRNA-treated group. **, $p < 0.01$ compared with induced and control miRNA-treated group. *D*, HD 16Q and HD 150Q cells were transiently transfected with control and E6-AP-miRNA1 plasmids as described in *A*. 24 h later, medium was replaced, and cells were differentiated with 5 mM dbcAMP and induced with 1 μM ponasterone A for 2 days. Cells were collected and subjected to immunoblot analysis using a p53 antibody.

miRNA-based knockdown system of E6-AP. In this system, the transient expression of E6-AP miRNA caused about 60–70% knockdown of endogenous E6-AP levels, whereas control miRNA had no effect on the level of E6-AP. The control and E6-AP miRNA constructs were transiently transfected to HD 150Q cells. Cells were then induced, and aggregate formation was monitored. The partial knockdown of E6-AP for 2 days caused a significant increase in the rate of aggregate formation (Fig. 8*A*) and increased accumulation of the high molecular weight complex of expanded polyglutamine proteins (Fig. 8*B*). Partial knockdown of E6-AP had a dramatic affect on expanded polyglutamine protein-induced cell death (Fig. 8*C*). However, knockdown of E6-AP also significantly induced the death of HD 16Q as well as wild-type neuro2a cells. How the E6-AP deficiency induces neuronal cell death is not clear at present. The p53 could be an important mediator, because, its level was found to be increased in the brain and prostate gland of E6-AP maternal-deficient mice (41, 44). The level of p53 was also increased in expanded polyglutamine protein-expressing cells (18). Now we have checked the level of p53 in E6-AP-deficient HD 16Q and HD 150Q cells. Our results show that partial knockdown of E6-AP caused increased accumulation of p53 in both HD 16Q and HD 150Q cells; however, its level was very high in E6-AP-deficient HD 150Q cells (Fig. 8*D*).

DISCUSSION

E6-AP is a HECT domain family E3 ubiquitin ligase identified on the basis of its ability to promote the degradation of p53 in association with papilloma virus E6 oncoprotein (37–40). Apart from p53, E6-AP also has been reported to degrade several other cellular substrates, including a human homologue of the yeast DNA repair protein RAD23, the multicopy maintenance protein-7 subunit involved in the initiation of DNA replication, Src family tyrosine kinase, epithelial cell transforming sequence-2 oncogene, and E6-AP, which is a target for itself (45–49). Here we demonstrate for the first time that the E6-AP is involved in proteasome-mediated degradation of misfolded polyglutamine proteins.

First, we have shown that the E6-AP interacts with soluble misfolded polyglutamine proteins and promotes their ubiqui-

tylation and degradation by the proteasome. E6-AP also recruits to polyglutamine aggregates in both cellular and transgenic mouse models. Secondly, we have found that overexpression of E6-AP reduces polyglutamine protein aggregation and polyglutamine protein-induced cell death, and this suppressive effect is more prominent when E6-AP is overexpressed along with the molecular chaperone Hsp70. The partial knockdown of E6-AP dramatically enhances the expanded polyglutamine protein-induced cell death with marginal increases in aggregation. Partial knockdown also promotes the death of normal glutamine repeat-expressing cells as well as wild-type neuro2a cells. There seems to be no correlation between aggregation and cell death in E6-AP-deficient expanded polyglutamine protein-expressing cells. Finally, we have demonstrated the increased expression of E6-AP in expanded polyglutamine protein-expressing cells as well as normal cells exposed to proteasomal stress.

E6-AP maternal-deficient mice (model mice for Angelman syndrome) exhibit severe cognitive and motor abnormalities without any gross structural alterations and neuronal loss in the hippocampus and cerebellum (41). An earlier study had shown that the SCA1 transgenic mice, deficient in E6-AP, demonstrated altered Purkinje cell morphology, accelerated SCA1 pathology, and reduced aggregate formation (22). But how loss of function of E6-AP accelerates SCA1 pathology and reduces the ataxin-1 aggregates in the Purkinje cells is not known. Their findings suggest that the severe pathological changes found in E6-AP-deficient SCA1 transgenic mice are most likely caused by the toxic effect of soluble mutant ataxin-1 aggravated by the lack of E6-AP function. Partial knockdown of E6-AP in our system also promoted expanded polyglutamine protein-induced cell death. However, E6-AP deficiency also promotes the death of normal glutamine repeat-expressing cells as well as wild-type neuro2a cells. This suggests that the loss of function of E6-AP might be up-regulating some of its target substrates, which is involved in neuronal cell dysfunction and death, and this situation is aggravated in the presence of expanded polyglutamine proteins. For example, E6-AP-deficient mice exhibit higher levels of p53 in the cerebellar Purkinje cells and p53, recently implicated in polyglutamine disease pathogenesis (22, 44, 50). Therefore, the expression of mutant ataxin-1 in the E6-AP-deficient Purkinje cells might result in severe stress and accelerated p53-induced neuropathology.

We have observed that the overexpression of E6-AP reduces, while partial knockdown increases polyglutamine protein aggregation. Because E6-AP promotes the degradation of soluble misfolded polyglutamine proteins, the results are quiet expected. However, our findings are in contrast with the results of Cummings *et al.* (22). How E6-AP deficiency reduces ataxin-1 aggregation in Purkinje cells is not clear presently. Interestingly, we have found that suppressive effects of aggregation and cell death mediated by E6-AP are more pronounced in the presence of Hsp70, suggesting a modulatory role of Hsp70 on the function of E6-AP and it may be *vice versa*. We have also noticed the interaction of E6-AP with Hsp70 in the co-immunoprecipitation assay (data not shown). It is possible that the E6-AP might be functioning as a cellular quality control ligase similar to CHIP. Therefore, critical concentrations of E6-AP and Hsp70 in cells might play a very important role in the regulation of polyglutamine protein aggregation.

Recently, the quality control E3 ligase CHIP has been demonstrated to play a vital role in the biology of neurodegenerative disorders involving protein misfolding including polyglutamine diseases. CHIP has been shown to reduce the aggregation and cell death mediated by mutant SOD1, α-synuclein, tau, and polyglutamine proteins (25–35). The HD transgenic mice, which are deficient in CHIP, display an accelerated disease phenotype while a higher level of expression of CHIP in the SBMA transgenic mouse model ameliorate the SBMA phenotype by enhancing the degradation of polyglutamine expanded androgen receptor (33, 34). Understanding the role of E6-AP as a cellular quality control ligase promises to be an exciting area of research.

We have also observed the recruitment of E6-AP to polyglutamine aggregates in both cellular and transgenic mouse models of HD. The recruitment of E6-AP or other E3 ligases and the proteasome to aggregates could be a desperate attempt by the cell to degrade aggregated protein by the proteasome. In fact, a conditional mouse model study showed that turning off the expression of mutant huntingtin led to reduction of aggregates and reversal of disease phenotype (23). Further study demonstrated that the reduction of aggregates is proteasome-dependent (24). Therefore, it is possible that the cells quality control system might be able to deal with the aggregated protein up to a certain level. Overburden of aggregated protein for prolonged time could ultimately collapse the quality control system (12–14). The overexpression of the quality control ligase could rescue cells by boosting the quality control system.

Another interesting observation is the increased expression of E6-AP in the expanded polyglutamine protein-expressing cells and cells treated with proteasome inhibitor. The expression of the Hsp70 chaperone is also increased in these cells, and E6-AP seems to follow a similar trend. The increase in the expression of E6-AP in stressed cells could also be an adaptive response similar to Hsp70 to get rid of misfolded and damaged proteins and suggests both E6-AP and Hsp70 might be working together to achieve this goal. Altogether, our studies provide evidence that E6-AP suppresses aggregation and toxicity of polyglutamine disease proteins by promoting their ubiquitylation and degradation. Thus, E6-AP overexpression might provide a new therapeutic approach for polyglutamine diseases.

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E6-AP Promotes Misfolded Polyglutamine Proteins for Proteasomal Degradation and Suppresses Polyglutamine Protein Aggregation and Toxicity

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