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# Regulation of Intracellular Accumulation of Mutant Huntingtin by Beclin 1\*

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Intracellular accumulation of mutant Huntingtin with expanded polyglutamine provides a context-dependent cytotoxicity critical for the pathogenesis of Huntington disease (Everett, C. M., and Wood, N. W. (2004) *Brain* 127, 2385–2405). Here we demonstrate that the accumulation of mutant Huntingtin is highly sensitive to the expression of *beclin 1*, a gene essential for autophagy. Moreover, we show that the accumulated mutant Huntingtin recruits Beclin 1 and impairs the Beclin 1-mediated long lived protein turnover. Thus, sequestration of Beclin 1 in the vulnerable neuronal population of Huntington disease patients might further reduce Beclin 1 function and autophagic degradation of mutant Huntingtin. Finally, we demonstrate that the expression of *beclin 1* decreases in an age-dependent fashion in human brains. Because *beclin 1* gene is haploid insufficient in regulating autophagosome function (Qu, X., Yu, J., Bhagat, G., Furuya, N., Hibshoosh, H., Troxel, A., Rosen, J., Eskelinen, E. L., Mizushima, N., Ohsumi, Y., Cattoretti, G., and Levine, B. (2003) *J. Clin. Invest.* 112, 1809–1820; Yue, Z., Jin, S., Yang, C., Levine, A. J., and Heintz, N. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 15077–15082), we propose that the age-dependent decrease of *beclin 1* expression may lead to a reduction of autophagic activity during aging, which in turn promotes the accumulation of mutant Htt and the progression of the disease.

Huntington disease (HD)<sup>3</sup> is one of at least nine inherited neurodegenerative disorders caused by a CAG repeat expansion in the corresponding causative genes (1). The gene involved in HD (*HD*) encodes a 350-kDa protein, named Huntingtin (Htt), that may function as a giant scaffold for organizing signaling complexes (4). The *HD* gene contains a highly polymorphic CAG repeat: the number of CAG copies ranges from 10 to 35 in unaffected individuals but exceeds 36 in patients with

HD. The expansion of CAG repeat in the mutant *HD* gene results in an extended polyglutamine (poly(Q)) stretch at the N terminus of the Htt protein. Expansion of the poly(Q) in Htt beyond 36 has complete penetrance in development of the disease. HD is characterized by the onset of progressive chorea, psychiatric symptoms, and dementia in early- to mid-adult life, which then follows a relentlessly downhill course with death usually occurring 12–15 years after the appearance of initial symptoms. Currently it is not clear what triggers the onset of HD, nor is there any effective treatment for this devastating disease.

Although the mechanism of neurotoxicity mediated by expanded poly(Q) is still highly controversial, it has been well established that expanded poly(Q) provokes a dominant gain-of-function neurotoxicity. The accumulation of expanded poly(Q)-containing proteins is a hallmark of HD as well as other poly(Q) expansion diseases (5–7). Accumulated truncated forms of Htt containing the N-terminal expanded poly(Q) are most easily recognized as neuronal inclusions (5–7). Treatment with Congo Red and trehalose, both of which reduce the accumulation of overexpressed expanded poly(Q)-positive proteins and increase the rate of their degradation, alleviated neuropathological findings and neurological symptoms in HD transgenic mouse models (8, 9). These results stress the importance of understanding the mechanism that regulates the turnover of proteins with expanded poly(Q).

Although neuronal Htt-N-terminal product inclusions are highly ubiquitinated, recent studies demonstrated that poly(Q) is a poor substrate for the eukaryotic proteasome (10). Thus, the highly ubiquitinated state of Htt inclusions may indicate the inability of the proteasomes in affected neurons to clear such abnormal truncated Htt proteins. On the other hand, there is pharmacological evidence to suggest the role of autophagy, a large scale catabolic mechanism, in regulating the degradation of the N-terminal part of Htt. The addition of 3-methyladenine, an inhibitor of Class III phosphatidylinositol 3-kinase and autophagy, has been shown to increase aggregate formation in x57 cells, a cell line derived from mouse striatal neurons, upon transfection with Htt-N-terminal products with expanded poly(Q) (11). Rapamycin, an inducer of autophagy, has been shown to reduce the aggregation of expanded poly(Q) polypeptides in transfected cells (12), protect against neurodegeneration in a fly poly(Q) peptide model of HD, improve the performance on behavioral tests, and decrease aggregate formation in a mouse transgenic N-terminal fragment model of HD (13). These results support a possible role of autophagy in regulating the turnover of expanded poly(Q) proteins. We still do not understand, however, the one or more molecular mechanisms that mediate the turnover of expanded poly(Q) proteins or whether a defect in such mechanism(s) might contribute to the age-dependent accumulation of Htt species.

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<sup>3</sup> The abbreviations used are: HD, Huntington disease; Htt, Huntingtin; poly(Q), polyglutamine; RIPA, radioimmune precipitation assay; siRNA, small interference RNA; WT, wild type; ST Q7/Q7, *STHdh*<sup>Q7/Q7</sup>; ST Q111/Q111, *STHdh*<sup>Q111/Q111</sup>; KD, knock down; Bcl-2 ID, Bcl-2-interacting domain; LC3, light chain 3; GFP, green fluorescent protein; TGN, trans-Golgi network.

Autophagy is an intracellular catabolic mechanism that mediates the degradation of large protein complexes and damaged organelles in a lysosome-dependent fashion (14–16). Although several forms of autophagy have been described, macroautophagy (referred to simply as ‘autophagy’ hereafter) is best studied. The hallmark of autophagy is the formation of a double-membrane cytosolic vesicle, the autophagosome, which sequesters cytoplasm and delivers it to the lysosomes for degradation (14). Elegant genetic studies have identified the genes and delineated pathways that regulate autophagy in yeast (17), although our knowledge of autophagy mechanisms in mammalian cells remains scanty (15, 16). Beclin 1 is a mammalian ortholog of Atg6/Vps30, a component of the Class III phosphatidylinositol 3-kinase complex in yeast that is essential for the formation of autophagosomes and cytosol-to-vacuole vesicles (18). Beclin 1 deficiency leads to impaired autophagy function in mammalian cells (2, 3, 19). Interestingly, the *beclin 1* gene is mono-allelically deleted in 40–75% of sporadic human breast and ovarian cancers (19), and heterozygous *beclin 1* mutant mice have increased spontaneous tumorigenesis (2, 3). Thus, the *beclin 1* gene encodes a haploid-insufficient tumor suppressor.

In this study, we show that the intracellular accumulation of Htt with expanded poly(Q) is highly sensitive to the expression levels of *beclin 1*. We show an age-dependent decline of *beclin 1* expression in human brains that might provide a mechanism for the accumulation of expanded poly(Q) species, perhaps contributing to the disease onset and disease progression of poly(Q) expansion diseases, including HD. Furthermore, we show that Beclin 1 is recruited to Htt inclusions in the R6/2 mouse brain and in the striatal samples of human HD patients. Thus, sequestration of Beclin 1 in the vulnerable neuronal population of HD patients might further reduce Beclin 1 function and autophagic degradation of expanded poly(Q)-containing Htt. Our study suggests a potentially important role of Beclin 1 in both the initiation and progression of HD.

## EXPERIMENTAL PROCEDURES

**Antibodies**—The commercial sources of antibodies were as follows: mouse monoclonal anti-HA (HA.11, Covance), mouse monoclonal anti- $\gamma$ -adaptn (BD Biosciences), 1C2 (Chemicon), and mouse monoclonal anti-Htt antibody (MAB2166, Chemicon).

**Beclin 1 Expression Analysis for Human Brain Samples**—The detailed protocols for DNA microarray analysis and quantitative reverse transcription-PCR are described elsewhere (38). For Western blot analysis, brain tissues samples were homogenized with a glass Dounce tissue grinder (Kontes) in RIPA-deoxycholate buffer (50 mM Tris buffer, pH 7.2, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, and 0.1% SDS) supplemented with protease inhibitors (Roche Applied Science) as well as phosphatase inhibitors (50 mM NaF, 5 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM NaVO<sub>4</sub>, and 1  $\mu$ M microcysteine).

**RNA Interference**—The following DNA sequences in the cDNAs of human and murine *Beclin 1* were used to produce double-stranded RNAs with a 3'-overhang: human Beclin 1, 5'-tgaggatgcagctgacag-3'; murine Beclin 1, 5'-cagtttggcacaatcaata-3'. In each case, the targeted sequence was separated from its reverse complement by a nine-nucleotide spacer (ttcaagaga) that served as the 3'-overhang. The constructs were annealed and inserted into the BglII and XhoI sites of pSRP retrovirus vector developed to express double-stranded RNAs bearing the 3'-overhang under the control of the H1 promoter. Pantropic retroviruses were made by transfecting 293T cells with each of the pSRP vectors harboring a Beclin 1 target sequence and plasmids containing the gag-pol-env constructs derived from Moloney leukemia virus. Virus-rich supernatants were used to infect cells, and infected cells were selected with puromycin (2–3  $\mu$ g/ml).

**Immunohistochemistry and Western Blotting**—8-week-old R6/2 and control mice (B6CBAF1/J) (Jackson Laboratory) were anesthetized with Forane (Abbott Laboratories) and transcardially perfused with 4% paraformaldehyde/phosphate-buffered saline for fixation. After cryoprotection with 20% sucrose/phosphate-buffered saline, 15- $\mu$ m-thick frozen sections were prepared. Paraffin-embedded striatal sections (thickness: 10  $\mu$ m) were obtained from normal controls ( $n = 3$ ; ages:  $87.7 \pm 1.5$  (mean  $\pm$  S.D.) years old; post-mortem periods:  $25.0 \pm 20.1$  h) and HD patients ( $n = 5$ ; ages:  $44.6 \pm 23.2$  years old; post-mortem periods:  $20.8 \pm 16.8$  h). Single immunostaining for Htt and Beclin 1 was performed using a Vectastain Elite ABC Kit (Vector Laboratories). Counterstaining was performed with 0.2% methyl green. For immunofluorescence double staining, EM48 and the rabbit polyclonal anti-Beclin 1 antibody (Santa Cruz Biotechnology) were used as primary antibodies, and fluorescein isothiocyanate-conjugated anti-mouse IgG antibody and rhodamine-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch) were used as secondary antibodies. For Western blotting, frozen human striatal tissue was homogenized in 7 volumes of 1% Triton X-100 buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, protease inhibitors). The homogenates were cleared by centrifugation ( $12,000 \times g$ , 15 min), and the supernatants were boiled after the addition of the sample buffer.

**Long Lived Protein Degradation Assay**—Cells were cultured with L-[<sup>3</sup>H]leucine (PerkinElmer Life Sciences) for 24 h and chased in media with nonradioactive leucine for another 24 h. Then the media was changed again and incubated for 4 more hours. The media were recovered and treated with 10% trichloroacetic acid to separate trichloroacetic acid-soluble (amino acids) and trichloroacetic acid-insoluble (proteins) fractions. The cells were completely dissolved with 0.2 N NaOH. Radioactivity was measured with a liquid scintillation counter (Beckman). Long lived protein degradation was calculated by dividing trichloroacetic acid-soluble radioactivity in the media by total radioactivity detected in the cells and media. The values were expressed as changes in -fold from the value obtained in normal control cells.

## RESULTS

**Intracellular Accumulation of Mutant Htt Is Sensitive to the Levels of Beclin 1**—To examine a possible role of Beclin 1 in regulating intracellular accumulation of Htt, we designed a hairpin siRNA to specifically reduce the expression of *beclin 1* (Becn1 siRNA) in a pair of striatal cell lines, *STHdh*<sup>Q7/Q7</sup> (ST Q7/Q7) and *STHdh*<sup>Q111/Q111</sup> (ST Q111/Q111). *STHdh*<sup>Q7/Q7</sup> (ST Q7/Q7) cells were derived from the striatal primordia of mice that were WT for *Hdh*<sup>Q7/Q7</sup>, encoding endogenous murine huntingtin with 7-glutamines; whereas *STHdh*<sup>Q111/Q111</sup> (ST Q111/Q111) cells are homozygous for a CAG repeat knock-in mutant allele *Hdh*<sup>Q111/Q111</sup>, which extends the poly(Q) segment of endogenous murine huntingtin to 111 residues (20). It has been shown previously that the endogenous mouse Htt Q7 protein in ST Q7/Q7 cells and the endogenous mutant Htt Q111 protein in ST Q111/Q111 cells are both soluble proteins (>350 kDa), except that the expanded poly(Q) tract in the Htt Q111 protein confers reduced mobility compared with the Htt Q7 protein on SDS-PAGE (20). Expression of Becn1 siRNA resulted in the reduction of Beclin 1 protein in both ST Q7/Q7 (18.8%) and ST Q111/Q111 cells (29.8%) as demonstrated by Western blotting followed by densitometric analysis (Fig. 1A). No effect of Becn1 knock down (KD) on the cell growth or gross morphology was detected (data not shown). Interestingly, Becn1 KD in ST Q111/Q111 cells led to a significant increase in the levels of Htt Q111 and the appearance of Htt Q111 species that migrated much slower as a smear-like immunoreactivity on SDS-PAGE, the latter of which suggested the formation of oligomeric

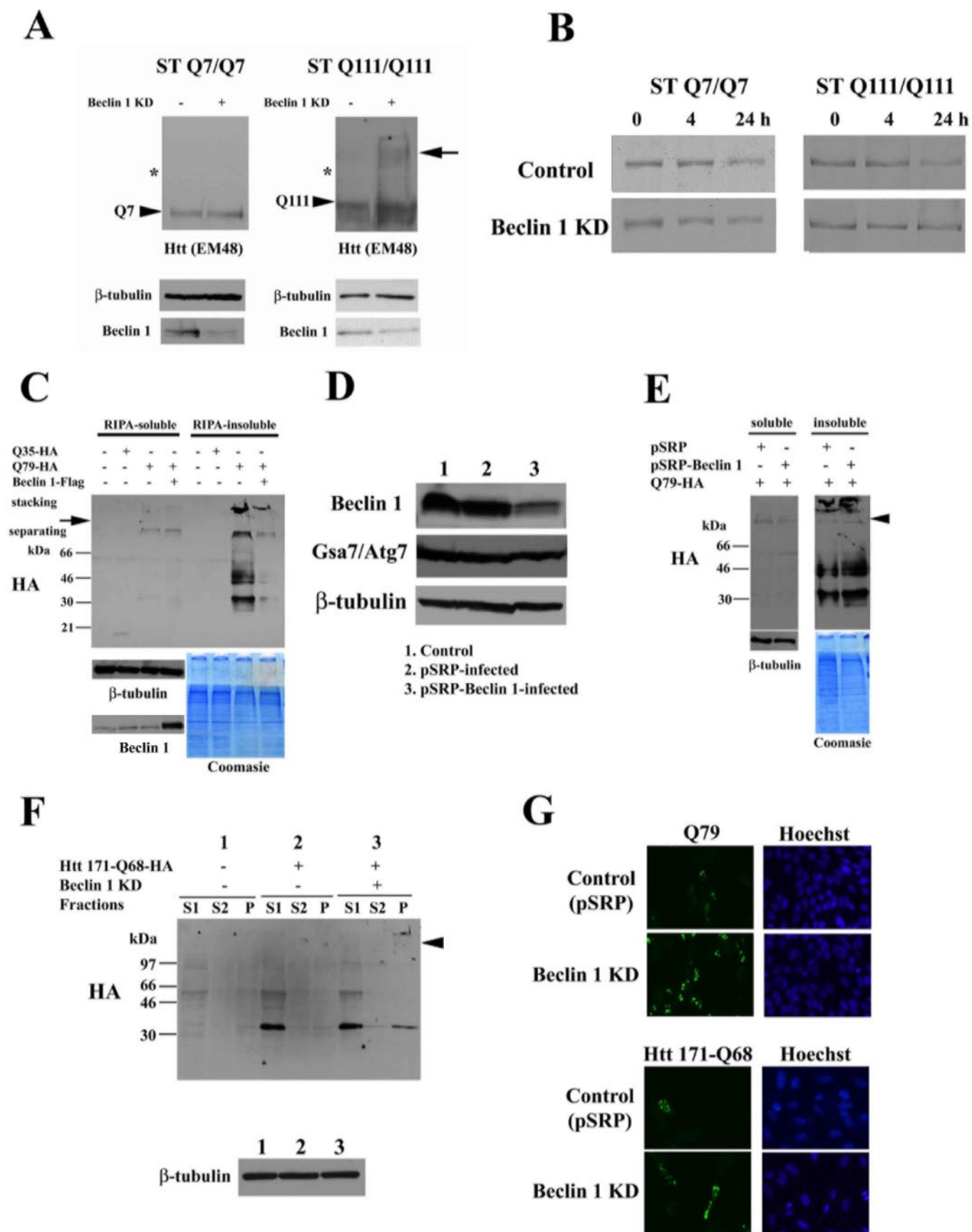
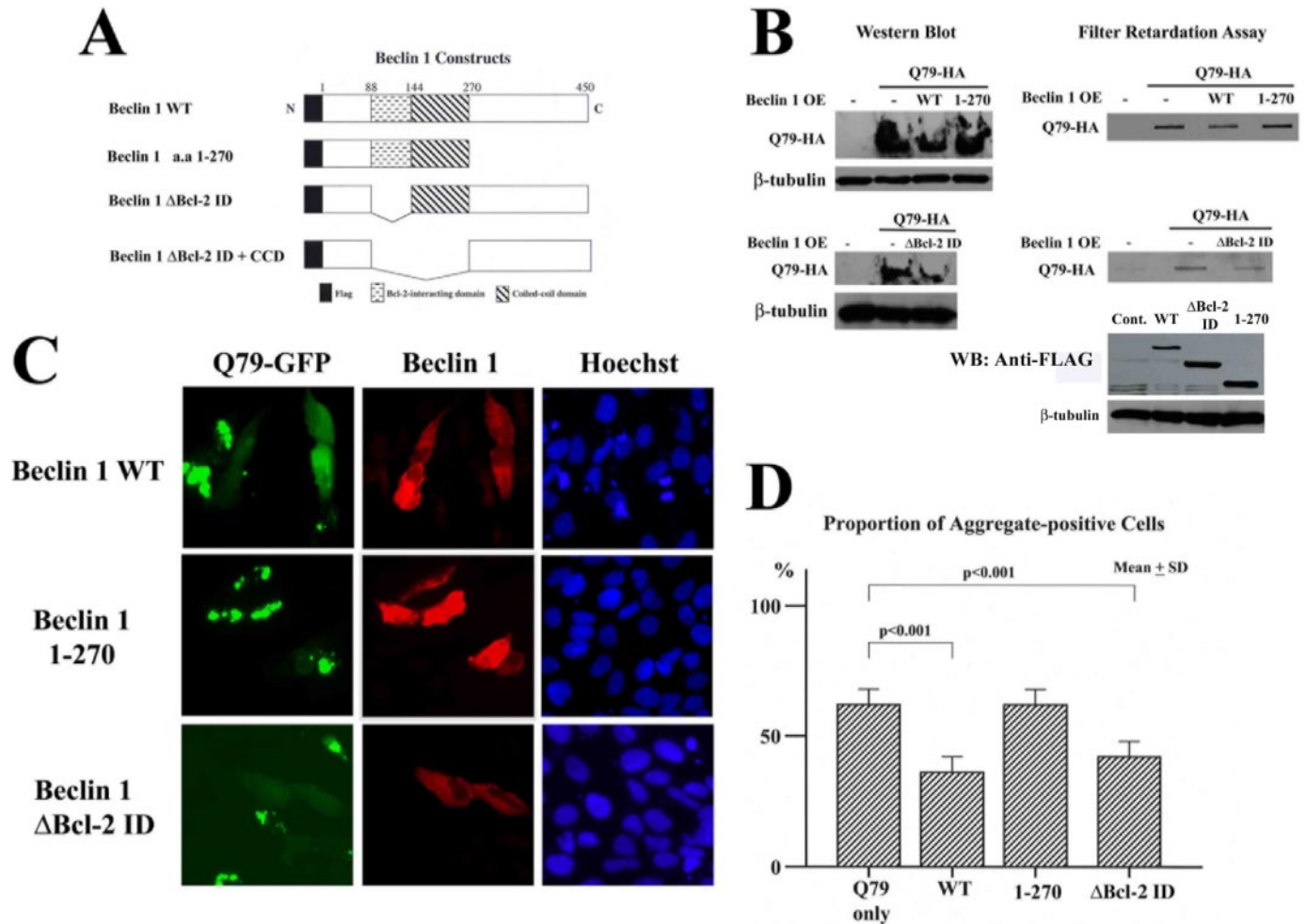


FIGURE 1. Intracellular accumulation of Htt with expanded poly(Q) is sensitive to the levels of *beclin 1* expression. **A**, striatal cell lines, ST Q7/Q7 and STQ111/Q111 (20), were infected with either a control vector (pSRP) or a vector expressing siRNA for Beclin 1 (Beclin 1 KD) and selected as pools in the presence of puromycin for 2 weeks. Western blots of cell lysate were probed with anti-Beclin 1 (lower panels) and EM48 (upper panels). Anti-β-tubulin (middle panels) was used as a loading control. The asterisks indicate the border between





**FIGURE 2. The Bcl-2-interacting domain of Beclin 1 is not required for reducing insoluble expanded poly(Q).** *A*, the constructs of Beclin 1 truncation mutants. *B* and *C*, H4 cells were transfected with expression vectors of Q79 and the full length or one of the Beclin 1 truncation mutants as shown in *A*. The effects of Beclin 1 truncation mutants on the accumulation of insoluble Q79 protein were examined by Western blotting and a filter retardation assay using anti-HA (*B*), or by anti-Beclin 1 immunostaining (*C*). The expression of the wild-type Beclin 1 and the Beclin 1 mutants were confirmed by Western blotting (*B*, lower). Nuclei were counterstained with Hoechst dye (*C*). OE, overexpression. *D*, proportions of wild-type and mutant Beclin 1-overexpressing cells with overt Q79 aggregation were counted. Four independent visual fields were analyzed in each group. The values for Q79 only, WT (wild-type), Bec 1-270, and ΔBcl-2 ID are 63.1 ± 6.1%, 34.9 ± 6.2%, 63.2 ± 6.1%, and 44.3 ± 5.2%, respectively. Statistical analysis was carried out by analysis of variance followed by Scheffe's post hoc tests.

Htt (Fig. 1A). In comparison, the reduction of *beclin 1* expression in ST Q7/Q7 cells did not cause the appearance of such smear-like immunoreactivity of WT Q7 Htt protein (Fig. 1A). This result indicates that the reduction of *beclin 1* expression led to the accumulation of Htt with expanded poly(Q). The slower migrating species of Htt in Q111/Q111 Becl1 KD cells, however, did not form aggregates visible by immunocytochemistry (data not shown). Thus, a reduction of *beclin 1* expression in Q111/Q111 cells leads to the formation of slower migrating Htt Q111 species on SDS-PAGE but not large aggregates.

To ask whether the accumulation of expanded poly(Q) in Q111/Q111 cells is caused by a reduction of poly(Q) turnover, we measured

the rate of Htt turnover in both Q7/Q7 and Q111/Q111 cells using a pulse-chase assay (Fig. 1B). In control vector-infected Q7/Q7 and Q111/Q111 cells, the levels of labeled Htt were significantly reduced as compared with the baseline level (0 h) after chasing for 24 h but not after 4 h (Q7: 91.8 ± 9.4% at 4 h, 51.0 ± 31.8% at 24 h; Q111: 86.2 ± 8.3% at 4 h, 44.4 ± 4.4% at 24 h, mean ± S.D.; Fig. 1B). In Becl1 KD Q7/Q7 cells, there was a time-dependent reduction of labeled Htt (72.9 ± 29.6% at 4 h, 35.2 ± 12.0% at 24 h; Fig. 1B). On the other hand, the levels of labeled mutant Htt in Becl1 KD Q111/Q111 cells after chasing for 24 h were not changed significantly compared with that of 4 h (70.2 ± 7.6% at 4 h, 79.1 ± 12.4% at 24 h; Fig. 1B). The difference in labeled Htt band

the stacking and separating gels. The *arrow* points to Htt smear-like immunoreactivity. The exposure time of films to ECL reagent-treated blot membranes to visualize EM48-reactive bands was 20 min. *B*, the cells were labeled with 0.2 mCi/ml [<sup>35</sup>S]Met for 20 min and chased in cold methionine media for the indicated hours. Htt was immunoprecipitated using anti-Htt antibody (MAB 2166) and analyzed by SDS-PAGE followed by autoradiography. *C*, H4 cells were transfected with expression vectors of Q35-HA, Q79-HA, or Q79-HA plus that of Beclin 1. Cell lysates were separated into RIPA-soluble and RIPA-insoluble fractions, and analyzed by Western blotting using anti-HA. Anti-β-tubulin and Coomassie Blue staining were used as loading controls for the RIPA-soluble and RIPA-insoluble fractions, respectively. Beclin 1 expression levels were monitored by Western blotting. The *arrow* indicates the border of the stacking and separating gels. *D*, H4 cells were infected with pSRP retrovirus or pSRP-Beclin 1 retrovirus and selected by puromycin for 2 days to establish pools stably expressing control vector or Beclin 1 siRNA. Western blots of cell lysates were probed with anti-Beclin 1, anti-human Gsa7/Atg7, or anti-β-tubulin. *E*, the accumulation of Q79-HA in control (pSRP-infected) and Becl1 KD H4 cells was evaluated as in *B*. The *arrowhead* indicates the border between the stacking gel and the separating gel. *F*, control pSRP-infected H4 cells or Becl1 KD H4 cells were transfected with an empty vector (pCDNA3-HA) or Htt 171-Q68-HA as indicated. The cells were extracted with RIPA buffer first, and the supernatants were designated as *S1*. The pellets were subsequently extracted with 1% Sarkosyl buffer. The resulting supernatants and pellets were designated as *S2* and *P*, respectively. The Western blot was probed with an anti-HA antibody. A Western blot of equal aliquots of the whole cell lysates before the extraction was probed with anti-β-tubulin to demonstrate the equal amount of starting material. *G*, control pSRP H4 and Becl1 KD H4 cells were transfected with expression vectors of Q79-HA or Htt 171-Q68-HA and immunostained by anti-HA 24 h after transfection. The nuclei were counterstained with Hoechst dye.

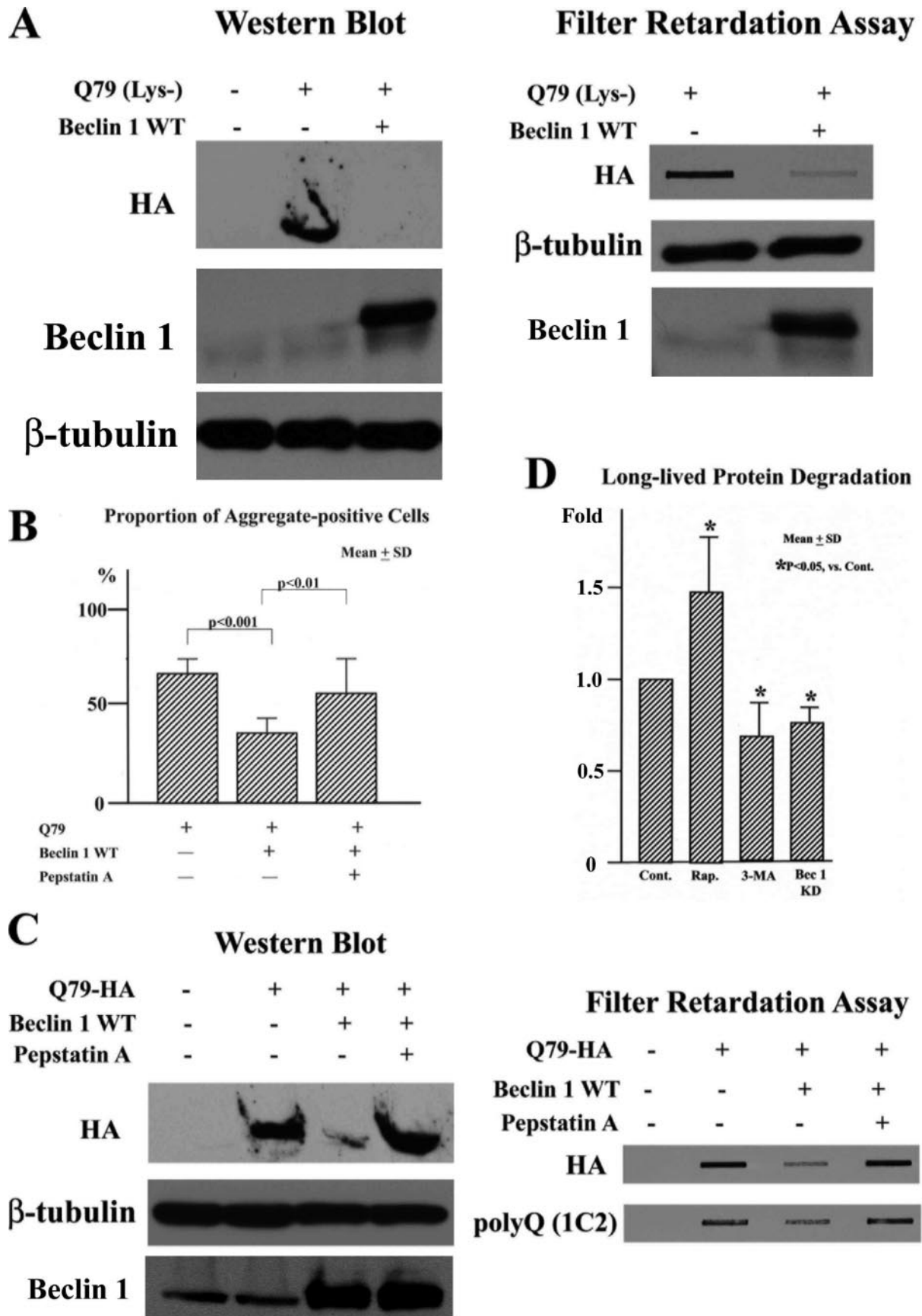


FIGURE 3. Intracellular accumulation of poly(Q) is sensitive to the levels of degradative autophagic function. *A*, H4 cells were transfected with a vector expressing Q79 Lys- with or without the wild-type Beclin 1 expression vector. The cells were processed for Western blotting (*left*) and filter retardation assay (*right*) using anti-HA to evaluate insoluble Q79 protein accumulation. The expression levels of Beclin 1 were evaluated by Western blotting. *B*, H4 cells were transfected with expression vectors of Q79 with or without Beclin 1 in the

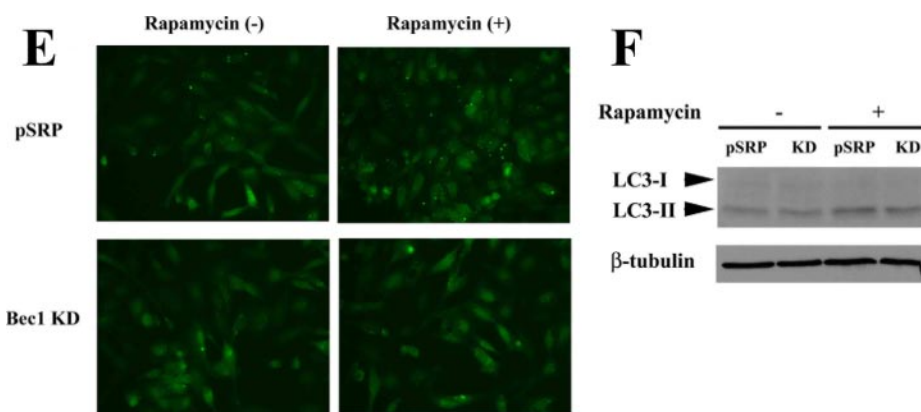


FIGURE 3—Continued

intensities at 24 h between Q7/Q7 and Q111/Q111 was statistically significant in Becl1 KD cells ( $p < 0.05$ ,  $t$  test). This finding suggests that Beclin 1-mediated long lived protein degradation pathway plays a critical role in the catabolism of mutant Htt.

To explore the mechanism by which Beclin 1 reduces the intracellular accumulation of mutant Htt with poly(Q), we chose the human neuroglioma-derived H4 cells, because they are more amenable to manipulation than the striatal cell lines. To validate the use of H4 cells as a system to characterize the mechanism of Beclin 1 in regulating the intracellular levels of Htt, we first determined if overexpression of *beclin 1* might cause changes in poly(Q) aggregation, as predicted by the results from the striatal cell lines described above. We compared the accumulation of insoluble aggregates in H4 cells transfected with expression vectors of Q79 and Q35 (21) with or without that of *beclin 1* (Fig. 1C). Q35 was identified as a 15-kDa single band present exclusively in the RIPA-soluble fraction, whereas most of Q79 was present in the RIPA-insoluble fraction, with a small portion migrating at 32 kDa and in the stacking gel in the RIPA-soluble fraction, consistent with the aberrant behavior of Q79 (Fig. 1C) (21). Overexpression of *beclin 1* led to a significant reduction of Q79 in the RIPA-insoluble fraction, whereas it had no effect on the levels of soluble Q79 (Fig. 1C).

To examine the effect of reducing *beclin 1* expression on the accumulation of insoluble poly(Q), we developed an siRNA retroviral vector (pSRP-Beclin 1) for human Beclin 1. pSRP-Beclin 1 specifically reduced the expression of *beclin 1*, whereas it had no effect on the expression level of human *gsa7/atg7* (Fig. 1D), a gene also involved in the execution of autophagy (22). An increased amount of RIPA-insoluble Q79 accumulated in Becl1 KD H4 cells *versus* control cells (Fig. 1E). Furthermore, the reduction of *beclin 1* expression in H4 cells also increased the amount of insoluble (P fraction) N-terminal truncated (amino acids 1–171) Htt with Q68 (Htt 171-Q68), which was present in the RIPA-soluble fraction in control H4 cells, and became partly RIPA-insoluble in Becl1 KD H4 cells (Fig. 1F).

To examine if a reduction in the solubility of expanded poly(Q) in Becl1 KD H4 cells was reflected in an increase in the formation of poly(Q) aggregates visible by immunocytochemistry, we transfected the expression vectors for Q79 and Htt 171-Q68 into control and Becl1 KD

H4 cells. The poly(Q) aggregates were visualized by immunostaining using anti-HA (Fig. 1G). In these cellular models, the poly(Q) aggregates were found in both the cytoplasm (~50%) and nuclei (~50%). We counted the percentages of cells with visible aggregates. The number of Q79 aggregates was significantly increased in Becl1 KD H4 cells in comparison with the control H4 cells ( $39.8 \pm 9.6$  (mean  $\pm$  S.D.)% in Becl1 KD *versus*  $23.1 \pm 4.1\%$  in control). Furthermore, while Htt 171-Q68 formed relatively few aggregates in control H4 cells, nearly double the number of Htt 171-Q68-aggregates was observed in Becl1 KD H4 cells ( $22.1 \pm 2.5\%$  in Becl1 KD *versus*  $12.1 \pm 5.2\%$  in control). These results suggest that Beclin 1 plays an important role in regulating the accumulation of proteins with expanded poly(Q) in H4 cells as well as in the striatal cell lines, providing a validation for the use of H4 cells as a model to explore the mechanism by which Beclin 1 regulates the accumulation of expanded poly(Q).

*The Bcl-2-interacting Domain of Beclin 1 Is Not Required for Reducing the Level of Expanded Poly(Q) Aggregates*—To explore the mechanism by which Beclin 1 reduces the accumulation of expanded poly(Q) aggregation, we characterized the domains of Beclin 1 that might be important for this function. Beclin 1 was identified as a Bcl-2-interacting protein (23). Beclin 1 contains a Bcl-2-interacting domain (Bcl-2 ID) and a coiled-coil domain. We constructed expression vectors for three Beclin 1 mutants, a C-terminal truncation containing amino acids 1–270 (Becl1–270), a Bcl-2-interacting domain ( $\Delta$ Bcl-2 ID) deletion mutant, and a Bcl-2 ID-coiled-coil domain deletion mutant (Fig. 2A). The Bcl-2 ID-coiled-coil deletion mutant could not be expressed in H4 cells, rendering further analysis of this mutant impossible. The effects of WT and mutant Beclin 1 proteins were compared in H4 cells cotransfected with Q79 expression vector and one of the other *beclin 1* expression vectors by measuring the levels of insoluble Q79 in the stacking gel and in a filter retardation assay (24). Overexpression of the N-terminal half of Beclin 1 (Becl1–270) failed to have any impact on the accumulation of Q79 (Fig. 2B). On the other hand, the deletion of Bcl-2-interaction domain retained the ability to reduce the insoluble poly(Q) accumulation. The effects of WT Beclin 1, Becl1–270 Beclin 1, and  $\Delta$ Bcl-2 ID Beclin 1 were further verified by immunostaining for Q79 aggregates. The expression of WT Beclin 1 and  $\Delta$ Bcl-2 ID Beclin 1 but not Becl1–270 Beclin 1

presence or absence of 10  $\mu$ M pepstatin A for 6 h before fixation. 48 h after transfection, the cells were processed for immunostaining using anti-HA. The numbers of cells bearing intracellular Q79 aggregates were counted, and expressed as mean  $\pm$  S.D. % of total cells. The cell counting was performed in five different visual fields for each group. Statistical analysis was carried out by analysis of variance and Scheffe's *post hoc* tests. The expression levels of Beclin 1 were shown by Western blotting. C, cell lysates were analyzed by Western blotting (left) and filter retardation assay (right) using anti-HA. The expression levels of Beclin 1 were evaluated by Western blotting. D, effects of *beclin 1* expression levels on long lived protein degradation. The rates of long lived protein turnover in H4 cells treated with 3-methyladenine (10 mM), rapamycin (*Rap.*, 1  $\mu$ M), stable Beclin 1 knockdown H4 cells (*Becl1 KD*), and H4 cells stably overexpressing HA-tagged Beclin 1 (*Becl1 ST*). The values were expressed as changes in-fold from the values obtained in normal control cells. Statistical analysis was performed by analysis of variance followed by Fisher's PLSD (protected least significant difference). The data were obtained from at least three independent experiments. E, reduction of *beclin 1* expression reduced GFP-LC3-positive autophagic vesicles. Control pSRP retrovirus-infected H4 cells and Becl1 KD H4 cells were transfected with GFP-LC3 for 24 h and then stimulated by rapamycin (1  $\mu$ M) or vehicle control for 4 h and examined by fluorescence microscopy. Cell counting procedures were carried out from three independent fields in each of two different experiments. F, reduction of *beclin 1* expression inhibits LC3-II formation induced by rapamycin. Control pSRP retrovirus-infected H4 cells and Becl1 KD H4 cells were stimulated by rapamycin for 4 h, and a Western blot of the cell lysates was probed with anti-LC3. Anti- $\beta$ -tubulin was used as a control.



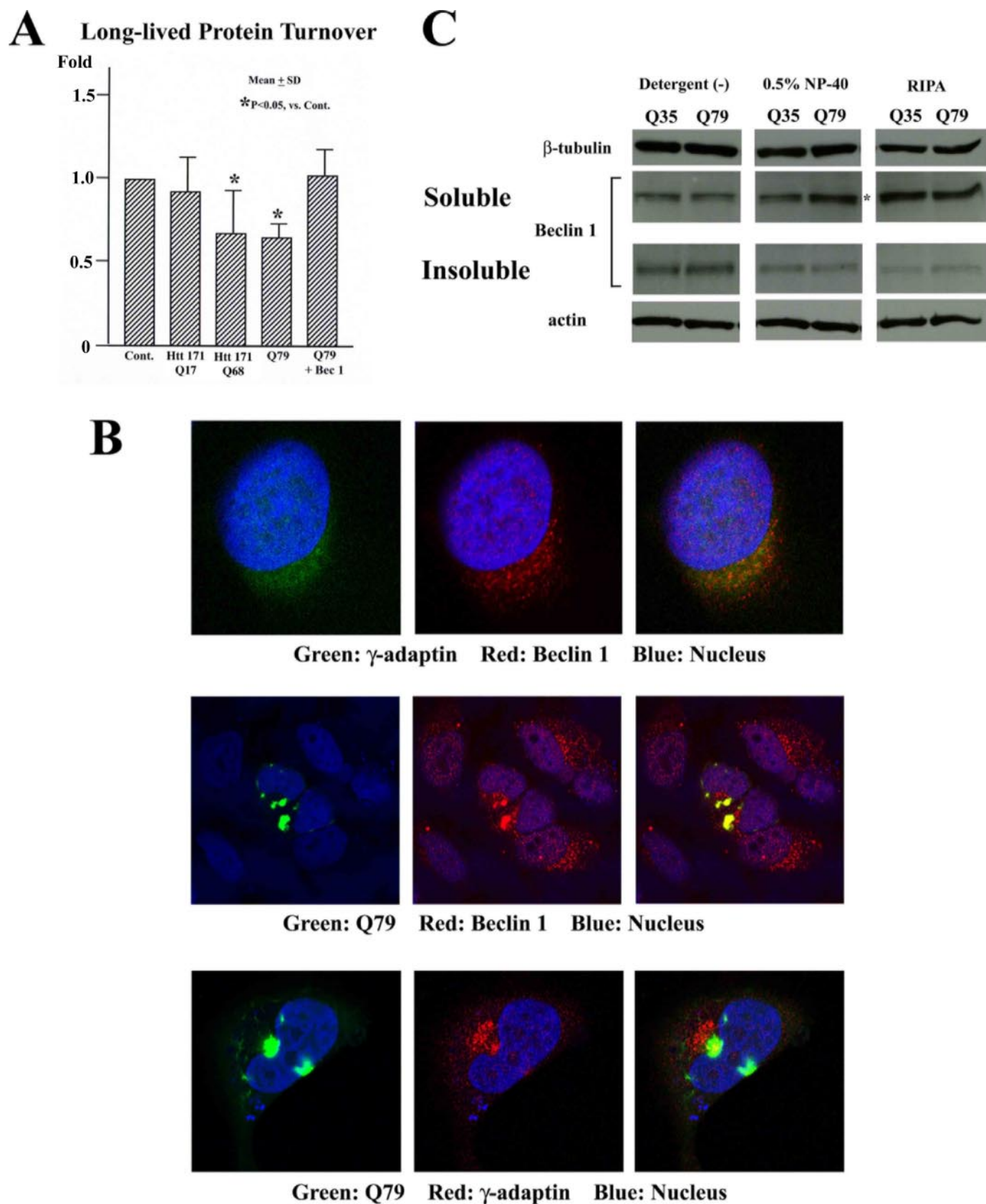
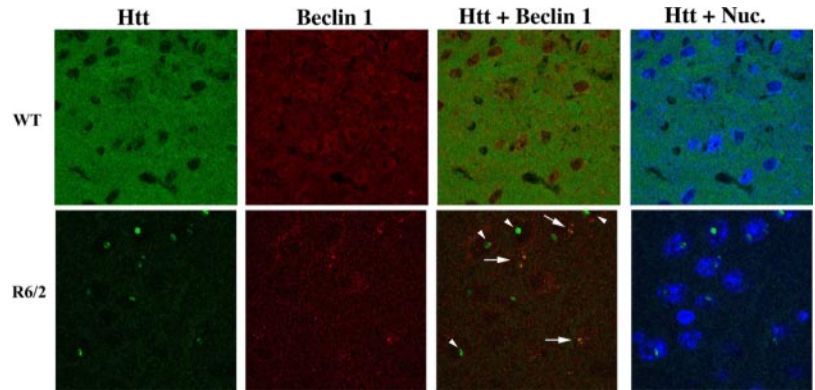


FIGURE 4. Effects of poly(Q) expression on long lived protein turnover and Beclin 1 distribution. A, H4 cells were transfected with expression vectors of Htt 171-Q17, Htt 171-Q68, Q79, or Q79 with that of *beclin 1*. Long lived protein degradation was measured as in Fig. 3D. B, the localization of Beclin 1 (red) and  $\gamma$ -adaptin (green) in control (top) and Q79-GFP-overexpressing (middle and bottom panels) H4 cells. Nuclei were counterstained by TO-PRO-3. The images were analyzed by confocal microscopy. C, solubility of Beclin 1 was assessed in Q35- and Q79-overexpressing cells. 48 h after transfection, the cells were extracted with a detergent-free buffer (150 mM NaCl, 20 mM Tris-Cl, pH 8.0), buffer with 0.5% Nonidet P-40, or RIPA. The levels of Beclin 1 in soluble and insoluble fractions were determined by Western blotting using anti-Beclin 1. The increased levels of Beclin 1 in Q79 expressing cells were marked with asterisk. The levels of  $\beta$ -tubulin and actin were used as loading controls.



**FIGURE 5. Beclin 1 is recruited to cytoplasmic Htt inclusions in R6/2 mouse brains.** Double immunostaining for Htt (EM48) and Beclin 1 in WT and R6/2 brain. Nuclear staining (*Nuc*) was performed with TO-PRO-3. The colocalization of Htt and Beclin 1 in R6/2 mouse brain sample was indicated with *arrows*. Nuclear aggregates without Beclin 1 recruitment are indicated with *arrowheads*. The images were analyzed by confocal microscopy. The image for EM48 (green) in the wild-type mouse brain section was exposed for longer time to reveal no significant immunoreactivity in the sample.



reduced the proportion of Q79-GFP aggregate-positive cells (Fig. 2, C and D). These results suggest that the activity of Beclin 1 in reducing the accumulation of expanded poly(Q) requires the C-terminal half of the protein (amino acids 271–450) but not the Bcl-2-interacting domain.

**Beclin 1 Reduces the Levels of Expanded Poly(Q) by Promoting Autophagy**—To exclude the possibility that the reduction in the intracellular accumulation of Q79 promoted by Beclin 1 might be in part due to the proteasome activity, we created a Q79 mutant (Q79 Lys<sup>-</sup>) that had its two Lys residues converted into Arg residues (Q79 K17R/K21R). Overexpression of *beclin 1* was still fully active in reducing the insoluble aggregates of Lys<sup>-</sup> Q79 as for those of Lys<sup>+</sup> Q79 (Fig. 3A). Because Lys residues are required for ubiquitination, this result suggests that Beclin 1 regulates the turnover of mutant Htt through an ubiquitination-independent pathway.

We reasoned if the ability of Beclin 1 to promote the turnover of mutant Htt is mediated through autophagy, its activity should be sensitive to inhibitors of lysosomal acid hydrolases, such as cathepsins, because the degradative power of autophagy relies on the fusion of autophagosomes and lysosomes (17). Hence, we examined whether the activity of Beclin 1 in reducing the accumulation of poly(Q) aggregates is sensitive to pepstatin A, an inhibitor of aspartic proteases, including cathepsin D. Overexpression of *beclin 1* in Q79-expressing H4 cells reduced the number of Q79 aggregate-positive cells ( $63.1 \pm 6.1$  (mean  $\pm$  S.D.)% versus  $34.9 \pm 6.2\%$ ), an effect inhibited by pepstatin A ( $55.1 \pm 17.7\%$ ) (Fig. 3B). Overexpression of *beclin 1* also reduced the accumulation of Q79 aggregate as shown by a reduction in the amount of poly(Q) aggregates in the stacking gel and in the filter retardation assay, which was also inhibited by pepstatin A (Fig. 3C). These results suggest that pepstatin A-sensitive aspartic proteases, such as cathepsin D, may be important for the degradation process promoted by Beclin 1. Moreover, 3-methyladenine also abolished the ability of overexpressed *beclin 1* to reduce Q79 aggregation, implying that the effect of Beclin 1 is dependent on phosphatidylinositol 3-kinase activity (data not shown).

Because autophagy has been proposed as one of the mechanisms involved in long lived protein turnover (25), we further measured the rates of long lived protein turnover in control H4 cells, and Becn1 KD H4 cells (Fig. 3D). Long lived protein degradation in each group was expressed as a relative value against that in the control H4 cells. Rapamycin, which stimulates autophagy (26), was used as a positive control; 3-methyladenine, which inhibits Class III phosphatidylinositol 3-kinase and autophagy (27), was used as a negative control. Consistent with our hypothesis, Becn1 KD resulted in a reduction of long lived protein degradation ( $0.746 \pm 0.118$ -fold). In comparison, 3-methyladenine inhibited long lived protein degradation ( $0.670 \pm 0.202$ -fold of the control level), whereas rapamycin enhanced it ( $1.477 \pm 0.298$ -fold) (Fig. 3D). These results indicate that long lived protein degradation is sensitive to

the reduction of *beclin 1* expression. Overall, these results support the role of Beclin 1-regulated long lived protein turnover in the catabolism of expanded poly(Q).

Beclin 1 has been shown to regulate autophagy in mammalian cells (19). To examine the effect of Beclin 1 on autophagosome formation in H4 cells, we first used the appearance of the phosphatidylethanolamine-conjugated form of microtubule-associated protein 1 light chain 3 (LC3-II) as a marker of autophagy activation. The microtubule-associated protein 1 light chain 3 (LC3-I) is a mammalian ortholog of Apg8/Aut7/Atg8, an essential protein for autophagy in yeast (28). LC3-II, a phosphatidylethanolamine-modified form of LC3-I, is specifically localized to the autophagosomal membrane, and the generation of LC3-II is an early and critical step in the formation of autophagosomes (28, 29). GFP-LC3 has been used as a marker for the intracellular levels of autophagy (15). We transfected H4 cells with an expression vector of GFP-LC3, and the levels of GFP-LC3-positive autophagosomes after rapamycin stimulation were determined by fluorescence microscopy (Fig. 3E). The levels of GFP-LC3-positive autophagosomes were significantly lower in Becn1 KD H4 cells than that of control (pSRP only,  $1.10 \pm 1.24$  (mean  $\pm$  S.D.); pSRP plus rapamycin,  $3.80 \pm 1.31$ ; Becn1 KD only,  $0.85 \pm 1.10$ ; Becn1 KD plus rapamycin,  $1.35 \pm 1.52$ ;  $p < 0.01$  pSRP plus rapamycin versus Becn1 KD plus rapamycin,  $n = 6$ ; \*,  $p < 0.01$  between pSRP plus rapamycin and Becn1 KD plus rapamycin, analysis of variance; Fig. 3E). We also determined the levels of LC3-II by Western blot and densitometric analysis (1647 for pSRP only, 1287 for Becn1 KD, 2315 for pSRP plus rapamycin, and 1947 for Becn1 KD plus rapamycin (arbitrary units); Fig. 3F). Although a significant amount of basal LC3-II was still present in Becn1 KD H4 cells, which is consistent with the fact that yeast atg6 is not required for Apg8-PE formation (31), the level of endogenous LC3-II after rapamycin stimulation was lower in Becn1 KD H4 cells than that of control H4 cells (Fig. 3F). From these findings, we concluded that the reduction of *beclin 1* expression in H4 cells led to a defect in autophagosome formation.

**Mutant Htt-N-terminal Fragments Have a Negative Effect on Beclin 1-mediated Protein Degradation**—Our studies demonstrate that a Beclin 1-regulated autophagy pathway plays an important role in regulating intracellular levels of Htt with expanded poly(Q). Because the accumulation of expanded poly(Q) is a hallmark of polyglutamine expansion diseases, we suspected that expanded poly(Q) might have a negative effect on Beclin 1-mediated protein turnover that in turn may promote and exacerbate the accumulation and oligomerization of expanded poly(Q). To examine this possibility, we first determined the rate of long lived protein turnover in H4 cells transfected with a control vector or expression vectors for Htt 171-Q17, Htt 171-Q68, and Q79 alone, or along with *beclin 1* expression vector. A significant reduction in the rate of long lived protein turnover was detected after the cells were trans-

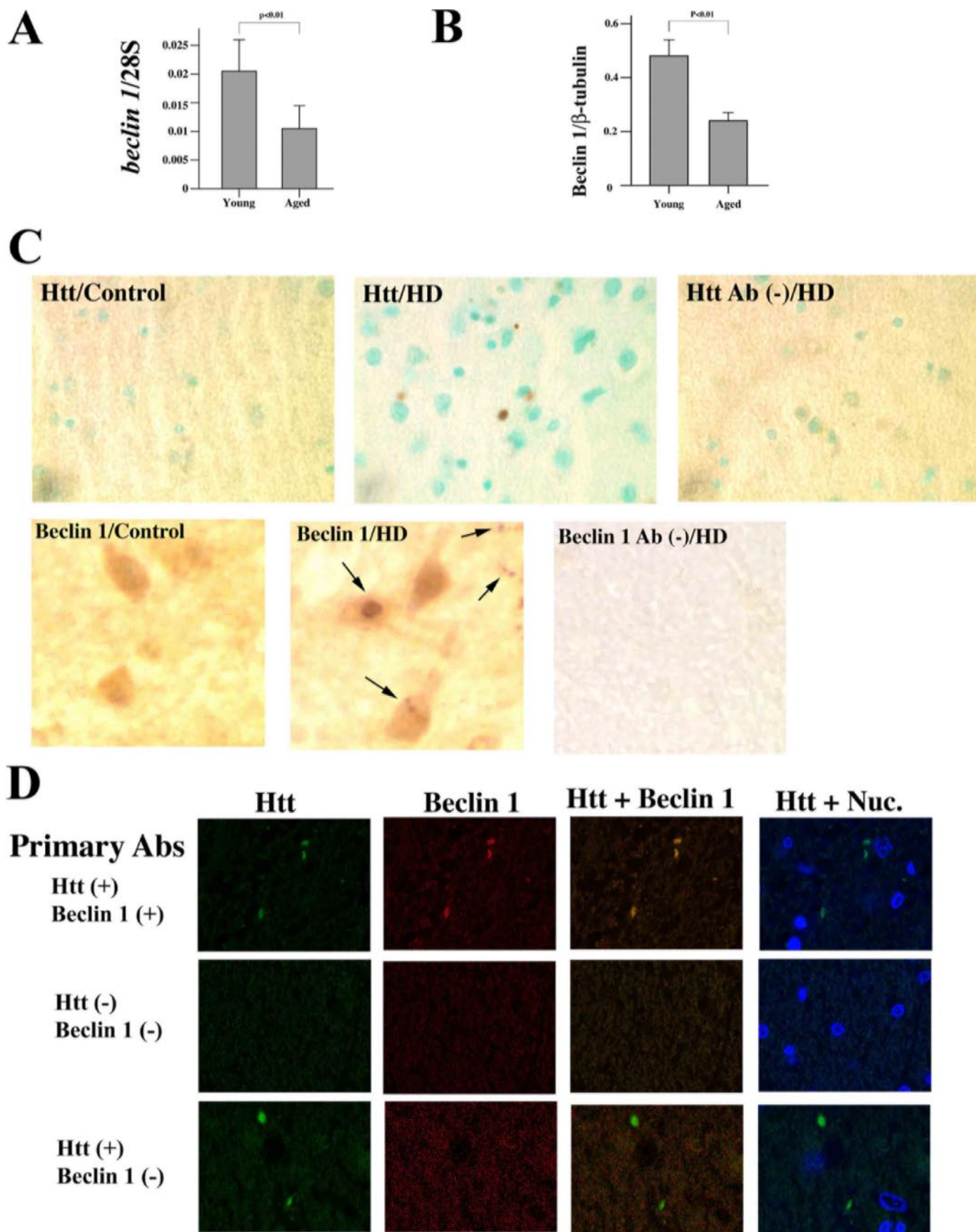


FIGURE 6. **Beclin 1 in human brain samples.** *A*, quantitative reverse transcription-PCR for *beclin 1* mRNA was performed for young ( $n = 6$ ,  $26.8 \pm 3.2$  (mean  $\pm$  S.D.) years old) and aged individuals ( $n = 6$ ,  $83.5 \pm 4.6$  (mean  $\pm$  S.D.) years old). The data were normalized by 28 S ribosomal RNA levels. *B*, Western blotting for Beclin 1 was performed for young ( $n = 6$ ,  $27.2 \pm 4.0$  (mean  $\pm$  S.D.) years old) and aged individuals ( $n = 7$ ,  $84.1 \pm 8.8$  (mean  $\pm$  S.D.) years old). The data were normalized by  $\beta$ -tubulin levels. In *C*, *Upper panels*, Htt was immunostained



ected with expression vectors of Q79 ( $0.629 \pm 0.085$  (mean  $\pm$  S.D.)-fold) and Htt 171-Q68 ( $0.638 \pm 0.262$ -fold), but not that of Htt171-Q17 ( $0.934 \pm 0.215$ -fold), at 24 h post-transfection, which was before any cell death could be detected (Fig. 4A). The negative effect of Q79 on the long lived protein turnover was fully corrected when cells were cotransfected with an expression vector of *beclin 1* (Fig. 4A). This effect of Beclin 1 is not likely due to a possible indirect influence on the cell survival, because no adverse effect of Q79 was detected in these cells 24 h after transfection; and furthermore, the same number of viable cells was used for comparison. These results suggest that the expression of expanded poly(Q) has a selective inhibitory effect on the long lived protein turnover regulated by Beclin 1.

To determine the mechanism by which expanded poly(Q) interferes with Beclin 1-sensitive long lived protein turnover, we examined the distribution of Beclin 1 in H4 cells. Consistent with a previous report that Beclin 1 was present predominantly in the *trans*-Golgi network (TGN) (32), we found that Beclin 1 was colocalized with  $\gamma$ -adaptin (Fig. 4B, upper panels) and giantin (data not shown), markers for the TGN in H4 cells. In H4 cells expressing Q79-GFP, however, Beclin 1 was partially re-distributed to Q79-GFP aggregates (Fig. 4B, middle panels). Moreover,  $\gamma$ -adaptin was also partially recruited to Q79 aggregates (Fig. 4B, lower panels). These observations suggest that Beclin 1, a TGN membrane-associated protein, may be translocated to Q79 aggregates along with a part of TGN itself. To examine whether the effect of expanded poly(Q) might have on the intracellular distribution of Beclin 1, we extracted H4 cells transfected with expression vectors of Q35 or Q79 with buffers with different amounts of detergents (Fig. 4C). The result indicated that an increased amount of Beclin 1 could be extracted with low detergent (0.5% Nonidet P-40)-containing buffer in H4 cells expressing Q79 than that in Q35-expressing H4 cells (the ratios of soluble/insoluble Beclin 1 corrected by tubulin and actin levels; 0.83 for Q35/no detergent, 0.53 for Q79/no detergent, 1.62 for Q35/0.5% Nonidet P-40, 3.37 for Q79/0.5% Nonidet P-40, 7.41 for Q35/RIPA, and 5.82 for Q79/RIPA). Thus, translocation of Beclin 1 to the areas associated with expanded poly(Q) aggregates made it become more amenable to the detergent extraction.

**Beclin 1 Is Recruited to the Cytoplasmic Htt Inclusions in R6/2 Mouse Brains**—The recruitment of Beclin 1 to poly(Q) aggregates in cultured cells prompted us to investigate if a similar phenomenon occurs in the brains of R6/2 mice, which exhibit aggregates formed from the HD exon 1 of Htt with 150–160 Qs (33). We examined the cerebral cortex of R6/2 mice by immunocytochemistry using EM48 (7). As previously reported (34), Htt aggregates in R6/2 mice were predominantly nuclear (Fig. 5). Intriguingly, anti-Beclin 1 immunoreactivity was associated with a subset of mutant Htt aggregates, which appears to be localized in the cytoplasm (arrows in Fig. 5, lower panel) but not those clearly in the nucleus (arrowheads in Fig. 5). This result suggests that Beclin 1 is recruited to a subset of the mutant Htt exon1 inclusions in R6/2 mice.

**Beclin 1 in Control and HD Post-mortem Human Brains**—Our above studies demonstrated that the intracellular accumulation mutant Htt is highly sensitive to the expression of *beclin 1*. To examine if a change in the expression levels of *beclin 1* might explain the age-dependent accumulation of mutant Htt, we determined the expression levels of *beclin 1* in the human prefrontal cortices from individuals ranging from 24 to 106 years of age. The age-dependent decline in *beclin 1* mRNA level was identified by quantitative reverse transcription-PCR: the levels of Beclin

1 mRNA were  $\sim$ 2-fold lower in older ( $83.5 \pm 4.6$  years old,  $n = 6$ ) versus young individuals ( $26.8 \pm 3.2$  years old,  $n = 6$ ) (Fig. 6A). To confirm this finding at the protein level, we performed Western blotting for Beclin 1 using the lysates prepared from the prefrontal cortex samples. In keeping with the quantitative reverse transcription-PCR data, Beclin 1 protein expression levels were also  $\sim$ 2-fold lower in the older ( $84.1 \pm 8.8$  years old,  $n = 7$ ) versus young individuals ( $27.2 \pm 4.0$  years old,  $n = 6$ ) (Fig. 6B). Because the autophagy is highly sensitive to the *beclin 1* gene dosage, our result suggests that the age-dependent reduction in the expression levels of *beclin 1* contributes to the accumulation of mutant Htt, which might have direct impacts to the disease onset and progression of HD.

To ask whether the accumulation of mutant Htt in human HD patients might have a negative impact on the Beclin 1 function as predicted from our studies using cultured models and animal models described above, we next studied the distribution of Beclin 1 in control and HD striatal samples and its relationship with that of Htt aggregates. We first performed diaminobenzidine staining for Htt in striatal sections from normal control brains ( $n = 3$ ) and HD brains ( $n = 5$ ) using EM48. Consistent with previous reports, punctate EM48-positive Htt inclusions were evident in all of the HD but none of the control brain samples examined (Fig. 6C, upper middle panel). Such staining was abolished after exclusion of the primary antibody (Fig. 6C, upper right panel). In contrast to the Htt inclusions in R6/2 mice (34), these inclusions were mostly extranuclear as reported previously (7).

Striatal neurons in the normal control brains showed diffuse immunoreactivity for Beclin 1 (Fig. 6C, lower left panel). On the other hand, inclusion-like deposits of Beclin 1-positive immunoreactivity were found in the HD samples. Most (>80%) of the inclusions seemed to be localized in the axon or neuropil, although we also observed intranuclear inclusions, which was consistent with a previous finding (7). Exclusion of Beclin 1 antibody did not yield this pattern of immunoreactivity on the section from the same subject (Fig. 6C, lower right panel). We also performed double fluorescence immunostaining for Htt and Beclin 1 in the striatal sections from HD brains. The results indicated that Beclin 1 was recruited to the mutant Htt-N-terminal inclusions in the striatal samples of HD patients (Fig. 6D). Taken together with our finding in R6/2 mice, our results indicate that Beclin 1 is recruited to the cytoplasmic Htt-N-terminal product aggregates in R6/2 mice as well as that in the striatum of HD patients.

## DISCUSSION

In this study, we provide evidence that Beclin 1 plays an important role in regulating intracellular levels of mutant Htt. The reduction of degradative autophagy function in Beclin 1 knockdown cells reduced the rate of long lived protein turnover and increased in the accumulation of mutant Htt. The accumulation of mutant Htt in ST Q111/Q111 cells induced by the partial inhibition of *beclin 1* expression supports the hypothesis that the age-dependent decline of *beclin 1* expression is a contributing factor to the age-delayed onset of HD. Furthermore, the ability of accumulated oligomeric mutant Htt to recruit Beclin 1 provides a mechanism to further exacerbate the age-dependent decline of autophagy function. Based on our findings, we conclude that the Beclin 1-regulated autophagy pathway mediates a part of intracellular long lived protein turnover that is critical for the catabolism of mutant Htt. The decline in the Beclin 1-mediated autophagy pathway may be critical

with EM48. EM48 was excluded from control. Methyl green was used for counterstaining. Lower panels, Beclin 1 immunostaining. In the middle image, inclusion-like deposits are indicated with arrows. In the right image, Beclin 1 antibody was excluded from the staining procedures. D, double immunostaining for Htt (green) and Beclin 1 (red) was performed on HD brain sections. Control immunostaining was carried out in the absence of one or both primary antibodies. Nuclei (Nuc) were counterstained by TO-PRO-3. Striatal sections were used for all the experiments in C and D.

## Beclin 1 in Huntington Disease

for both the onset as well as the progression of HD. A recent study showed that the liver-specific knock-out of Atg7, another autophagy-related gene, leads to an increase in the propensity of protein aggregation in the liver (35). Along with this study, our results emphasize the importance of autophagy-mediated protein degradation machinery for suppressing aberrant protein aggregation.

The effect of *beclin 1* gene knockdown by siRNA on the intracellular accumulation of mutant Htt but a much milder effect on that of WT Htt suggests at least two mutually nonexclusive possibilities regarding the catabolism of WT and mutant Htt. First, the increased accumulation of mutant Htt in Beclin 1 KD cells may indicate the exclusive dependence of mutant Htt degradation on the autophagy pathway, whereas the degradation of WT Htt may be carried out by multiple pathways. This is consistent with the fact that Beclin 1 knockdown only had a partial effect in inhibiting the long lived protein turnover. Second, the excessive accumulation of mutant Htt in Beclin 1 knockdown cells may be in part due to the oligomerization of mutant Htt which in turn further reduces its own degradation. This is supported by our demonstration that Beclin 1 is recruited to the aggregated mutant Htt in cultured cells, HD transgenic mouse models, and the striatal samples from HD patient. Our studies demonstrate a critical role of Beclin 1-mediated long lived protein degradation in the accumulation of mutant Htt. Based on our studies using the Beclin 1 mutants, the C-terminal half of the molecule is essential for the execution of autophagy. As this part of Beclin 1 is important for the association with cellular membranes (36), it is deduced that Beclin 1 should be tethered to cellular membranes to act in the autophagy process. Our result shows that the Bcl-2 ID is not indispensable for the execution of autophagy, which is consistent with a recent report demonstrating that the Bcl-2 ID is important for functional modulation but not essential for the execution of autophagy (37).

Our study provides a potential mechanism that explains the age-delayed onset of HD. Because the age-dependent decline in the levels of *beclin 1* mRNA in human brains is largely consistent with that of Beclin 1 protein, we conclude that the reduction of *beclin 1* expression is mostly due to the reductions in *beclin 1* transcription. Recently, Lu *et al.* (38) defined a set of genes whose transcriptions were reduced in human frontal cortex after the age of 40 years. Lu *et al.* (38) further demonstrated that the promoters of the genes with reduced expression were preferentially damaged by oxidative stress with reduced base-excision DNA repair. Our result raises the possibility that the promoter of *beclin 1* gene may be sensitive to DNA damage. The age-dependent reduction of *beclin 1* expression would in turn contribute to the age-dependent decline of autophagy function (39, 40) and the consequential accumulation of mutant Htt which eventually leads to the onset of disease.

Mutant Htt aggregates have been shown to recruit many different proteins *in vivo* and in cultured cells (30). In most cases, the recruited proteins became tightly associated with the detergent-insoluble aggregates. The recruitment of Beclin 1 by the mutant Htt-N-terminal fragments, however, appears to be very different from most proteins known to be recruited to mutant Htt aggregates, because the recruited Beclin 1 became detergent-soluble in our case. Although most of the endogenous Beclin 1 immunoreactivity in mutant Htt-expressing cells was around poly(Q) aggregates, we could not demonstrate significant association between Beclin 1 and truncated Htt proteins (Htt 480-Q17 and Htt 480-Q68) by a coimmunoprecipitation assay (data not shown). Instead, we found that Beclin 1 appeared to be recruited to poly(Q) aggregates along with at least a part of the TGN itself. Mislocalization of Beclin 1 as well as distortion of TGN may together result in the inhibition of autophagy function in mutant Htt-expressing cells.

In summary, we demonstrate the critical role of Beclin 1 in mediating the catabolism of mutant Htt. Our study pointed out that the age-dependent decline of *beclin 1* expression and possible associated reduction of Class III phosphatidylinositol 3-kinase activity is critical for the decline of autophagic function, which may lead to the accumulation of mutant Htt and the age-delayed disease onset of HD. Furthermore, the ability of accumulated mutant Htt to recruit Beclin 1 provides a mechanism to further reduce the function of Beclin 1 and exacerbate the disease progression. Our study suggests that pharmacological means to increase of Beclin 1 function may be beneficial in delaying the onset as well as slowing the progression of HD.

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*Addendum*—While we were preparing this manuscript, Iwata *et al.* reported that autophagy plays an important role in clearing cytoplasmic Htt aggregates. Our study strengthens the importance of autophagy by demonstrating the role of Beclin 1, an autophagy gene, in the pathophysiology of HD.

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**Regulation of Intracellular Accumulation of Mutant Huntingtin by Beclin 1**  
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