THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 17, pp. 11318–11325, April 24, 2009
© 2009 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

SCAMP5 Links Endoplasmic Reticulum Stress to the Accumulation of Expanded Polyglutamine Protein Aggregates via Endocytosis Inhibition*[S]

Received for publication, October 2, 2008, and in revised form, January 12, 2009 Published, JBC Papers in Press, February 24, 2009, DOI 10.1074/jbc.M807620200

Jee-Yeon Noh[‡§], Huikyong Lee[§1], Sungmin Song[§], Nam Soon Kim[¶], Wooseok Im[‖], Manho Kim[‖], Hyemyung Seo[**], Chul-Woong Chung[‡], Jae-Woong Chang[§], Robert J. Ferrante[‡‡], Young-Jun Yoo[‡], Hoon Ryu[‡‡], and Yong-Keun Jung[§2]

From the [‡]Department of Life Science, Gwangju Institute of Science and Technology, Gwangju 500-712, Korea, [§]Creative Research Initiative-Acceleration Research, School of Biological Science, Seoul National University, Seoul 151-742, Korea, the [¶]Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-600, Korea, the [‖]Department of Neurology, Seoul National University Hospital, Seoul 110-744, Korea, the [**]Department of Molecular and Life Science, Hanyang University, Gyeonggi-do 426-791, Korea, and the [‡‡]Department of Neurology, Boston University School of Medicine, Bedford, Massachusetts 01730

Accumulation of expanded polyglutamine proteins is considered to be a major pathogenic biomarker of Huntington disease. We isolated SCAMP5 as a novel regulator of cellular accumulation of expanded polyglutamine track protein using cell-based aggregation assays. Ectopic expression of SCAMP5 augments the formation of ubiquitin-positive and detergent-resistant aggregates of mutant huntingtin (mtHTT). Expression of SCAMP5 is markedly increased in the striatum of Huntington disease patients and is induced in cultured striatal neurons by endoplasmic reticulum (ER) stress or by mtHTT. The increase of SCAMP5 impairs endocytosis, which in turn enhances mtHTT aggregation. On the contrary, down-regulation of SCAMP5 alleviates ER stress-induced mtHTT aggregation and endocytosis inhibition. Moreover, stereotactic injection into the striatum and intraperitoneal injection of tunicamycin significantly increase mtHTT aggregation in the striatum of R6/2 mice and in the cortex of N171-82Q mice, respectively. Taken together, these results suggest that exposure to ER stress increases SCAMP5 in the striatum, which positively regulates mtHTT aggregation via the endocytosis pathway.

The expansion of CAG repeats (usually beyond a critical threshold of ~37 glutamine repeats) encoding polyglutamine (polyQ)[3] causes, to date, nine late-onset progressive neurodegenerative disorders (1, 2). Expanded polyQ-containing huntingtin is the main aggregate component in the affected neurons (3). Also, molecular chaperones, such as Hsp70, Hsp40/HDJ1 (dHDJ1), and chaperonin TRiC, perturb the aggregation of polyQ track protein and reduce polyQ track cytotoxicity in yeast and cell lines (4–6) and in *Drosophila* and mouse models (4, 7). Thus, it seems that HD pathology is closely correlated with the accumulation of insoluble aggregates of mutant huntingtin (mtHTT) containing expanded polyQ (2, 3, 8, 9).

Endoplasmic reticulum (ER) stress is crucial in many biological responses and is generated by various signals, such as unfolded protein response, aberrant calcium regulation, oxidative stress, and inflammation (10, 11). ER stress response is generally considered an adaptive reaction of cells to environmental stress, serving as a survival signal (10). On the other hand, increasing evidence also strengthens the importance of ER stress in human diseases. A malfunction or excess of ER stress response caused by aging, genetic mutations, and environmental insults is implicated in human diseases, such as Alzheimer disease, Parkinson disease, diabetes mellitus, and inflammation (12–16). mtHTT also induces ER stress at the early stage of HD, and pathogenic ER stress from an aging or stressful environment is severe at the late stage of HD (17–19). However, the molecular event linking the aggregation of polyQ track protein to ER stress response is unknown.

The ubiquitin/proteasome pathway, a major protein degradation system, is altered or impaired in the cell culture model of HD (20–22). On the contrary, autophagy employing lysosomal degradation has been recently considered as a major clearance pathway of insoluble aggregates of polyQ track protein. Thus, inhibition of autophagy has been suggested to modulate the aggregate formation of mtHTT and to affect the toxicity of polyglutamine expansions in fly and mouse models of HD (23–25). However, a key molecule controlling the aggregation and clearance of polyQ track proteins needs to be identified.

To further our understanding of the regulation of polyQ track protein aggregation, we screened human full-length cDNAs and isolated SCAMP5 (secretory carrier membrane protein 5) as a modulator of polyQ track protein aggregation.

* This work was supported in part by Creative Research Initiative Grant R17-2008-038-01000, the Brain Research Center of the 21st Century Frontier Research Program, and Ubiquitom projects funded by the Korea Science and Engineering Foundation of the Korean Government (Ministry of Education and Science Technology).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2 and additional references.

[1] Supported by a Korea Research Foundation program.

[2] To whom correspondence should be addressed: School of Biological Science/Bio-MAX Institute, Seoul National University, Gwanakro-599, Seoul 151-742, Korea. Tel.: 82-2-880-4401; Fax: 82-2-873-7524; E-mail: ykjung@snu.ac.kr.

[3] The abbreviations used are: polyQ, polyglutamine; ER, endoplasmic reticulum; mRFP, monomeric red fluorescent protein; GFP, green fluorescent protein; HD, Huntington disease; mtHTT, mutant huntingtin; PBS, phosphate-buffered saline; RT, reverse transcription.

SCAMP5 is up-regulated by mtHTT and ER stress and functions to inhibit endocytosis to increase mtHTT aggregation.

EXPERIMENTAL PROCEDURES

Human cDNA Collections—Total RNAs were purified from neuronal cells and tumor samples. The full-length cDNA library was constructed by PCR-based oligocapping methods (45) and subcloned into the pCNS-D2 mammalian expression vector. Individual cDNA was identified by DNA sequencing analysis and annotated. A group of 2,200 cDNA clones including 600 kinase cDNAs was selected, and another group of 2,000 cDNA clones in pCMV6 was purchased from OriGene, Inc.

Plasmid Constructions—Human SCAMP5 cDNA was amplified by PCR and subcloned into the HindIII site of pEGFP-N1 (pGFP-S5), pcDNA-HA (pSCAMP5-HA), or pDsRed-Monomer-C1 (pmRFP-S5; Clontech) in a sense or antisense orientation. Green fluorescent protein (GFP)-fused polyglutamine ($n = 110$) (p110Q-GFP) was constructed by subcloning the PCR product into pEGFP-C1. Deletion mutants (D1–D4) were constructed by subcloning of the PCR products containing respective amino acid residues of human SCAMP5 into pcDNA-HA and pEGFP-N1. Human SCAMP1 cDNA was amplified by PCR and subcloned into the KpnI site of pcDNA-HA (pSCAMP1-HA). The pSCAMP1-(1–149)/5 chimera was constructed by subcloning the PCR products of the SCAMP5 and SCAMP1 fragment (1–447 bp) into the KpnI and HindIII sites of pcDNA-HA.

Cell-based Functional Screening—For primary screening, HEK293F cells grown in a 96-well culture plate were co-transfected with pDsRed-Monomer-C1, pHTTex120Q-GFP (or p110Q-GFP), and an individual cDNA in a mammalian expression vector. GFP-positive cells were observed for 18, 24, and 36 h under a fluorescence microscope for the aggregation of polyQ track protein. Transfection efficiency was normalized by that of RFP. For a secondary screening, the putative positive clones were examined again for their overexpression and knockdown effects on the aggregation of polyQ track protein.

Primary Culture of Striatal, Cortical, and Hippocampal Neurons—The striatal, cortical, and hippocampal tissues of rat embryonic day 16 brain were dissociated by incubating with 0.01% trypsin/EDTA (Invitrogen) and plated on culture dishes coated with poly-L-lysine (0.01% in 100 mM borate buffer, pH 8.5) (Sigma) in Neurobasal™ medium containing 2% B27 supplement (Invitrogen). Neurons grown *in vitro* for 3 days were transfected with the appropriate DNA using Lipofectamine™ 2000 reagent (Invitrogen).

Immunocytochemistry—Immunocytochemistry was performed as described (32). Briefly, rat primary neurons were grown on a coverslip coated with poly-L-lysine, fixed with 4% paraformaldehyde, and permeabilized with 0.01% Triton X-100 (Sigma). After blocking with 3% bovine serum albumin, the samples were incubated with anti-SCAMP5 (Abcam), anti-CHOP (Santa Cruz Biotechnology), or anti-ubiquitin antibody (Zymed Laboratories, Inc. and Chemicon) at room temperature for 1 h. After reaction with secondary antibodies (Molecular Probes), samples were examined with an UltraVIEW confocal imaging system (PerkinElmer Life Sciences) and an Eclipse TE 2000-U microscope (Nikon).

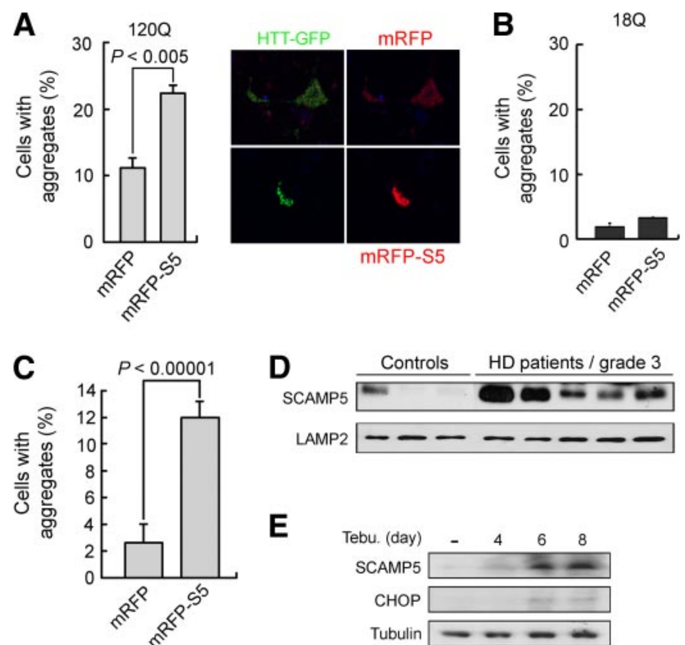

FIGURE 1. **Up-regulation of SCAMP5 in the striatum of HD patients and overexpression effects on the aggregation of mtHTT in cultured cells.** *A*, SCAMP5 increases the aggregation of mtHTT. Rat primary striatal neurons were cultured from embryonic day 16 and maintained for 3 days *in vitro* as described under "Experimental Procedures." The striatal neurons were then co-transfected with HTTex120Q-GFP (120Q/HTT-GFP) and either mock (mRFP) or SCAMP5 fused with monomeric RFP (mRFP-S5; *red*) for 24 h and observed under a confocal microscope (*right panel*). Percentages of aggregation were determined by counting cells showing the aggregation of HTTex120Q-GFP among total GFP-positive cells (350 cells/counting). *Bars* represent means ± S.D. ($n = 3$) (*left panel*). *B*, SCAMP5 does not affect the aggregation of huntingtin exon 1 containing the normal range of polyQ ($n = 18$). SH-SY5Y cells were co-transfected with HTTex18Q-GFP (18Q) and either mRFP or mRFP-S5 for 24 h. Cells were then examined under a microscope as in *A*. *C*, PC12 rat neuronal cells stably expressing mutant huntingtin (HTT 103Q-GFP) under the control of ecdysone-inducible promoter were pre-incubated for 18 h with 2 μM tebufenozide. Cells were then co-transfected for an additional 96 h with either mock (mRFP) or mRFP-S5 in the presence of tebufenozide and examined under a fluorescence microscope. Cells harboring the aggregates of HTT 103Q-GFP were examined as in *A*. *D*, a Western blotting showing up-regulation of SCAMP5 in HD patients is shown. Striatum samples from five HD patients (grade 3) and three age-matched controls were analyzed with Western blotting using the indicated antibodies. *E*, PC12 rat neuronal cells stably expressing mutant huntingtin (HTT 103Q-GFP) under the control of an ecdysone-inducible promoter were incubated for the indicated times with 2 μM tebufenozide (*Tebu.*). Thereafter, cells were lysed and analyzed by Western blotting using anti-SCAMP5 and anti-CHOP antibodies.

Immunohistochemistry—Sectioned brain tissues were permeabilized with 0.3% Triton X-100 in phosphate-buffered saline (PBS) for 30 min and blocked with 5% bovine serum albumin in PBS for 30 min. Thereafter, the tissues were incubated with anti-mtHTT antibody (EM48, Chemicon) and anti-SCAMP5 or anti-GRP78 antibody (Santa Cruz Biotechnology) overnight at 4 °C. The samples were washed with PBS and incubated with secondary antibody and Hoechst 33342 (Molecular Probes).

Western Blotting—Protein samples in buffer (10% glycerol, 2% SDS, 62.5 mM Tris-HCl, 2% β-mercaptoethanol, pH 6.8) were separated by SDS-PAGE and subjected to Western blotting as described (39). For preparation of insoluble fractions, cell lysates were subjected to centrifugation at 400 × *g* for 3 min. The pellets were washed with lysis buffer (0.1% Nonidet P-40, 250 mM NaCl, 5 mM EDTA, 50 mM Hepes, pH 7.4) and

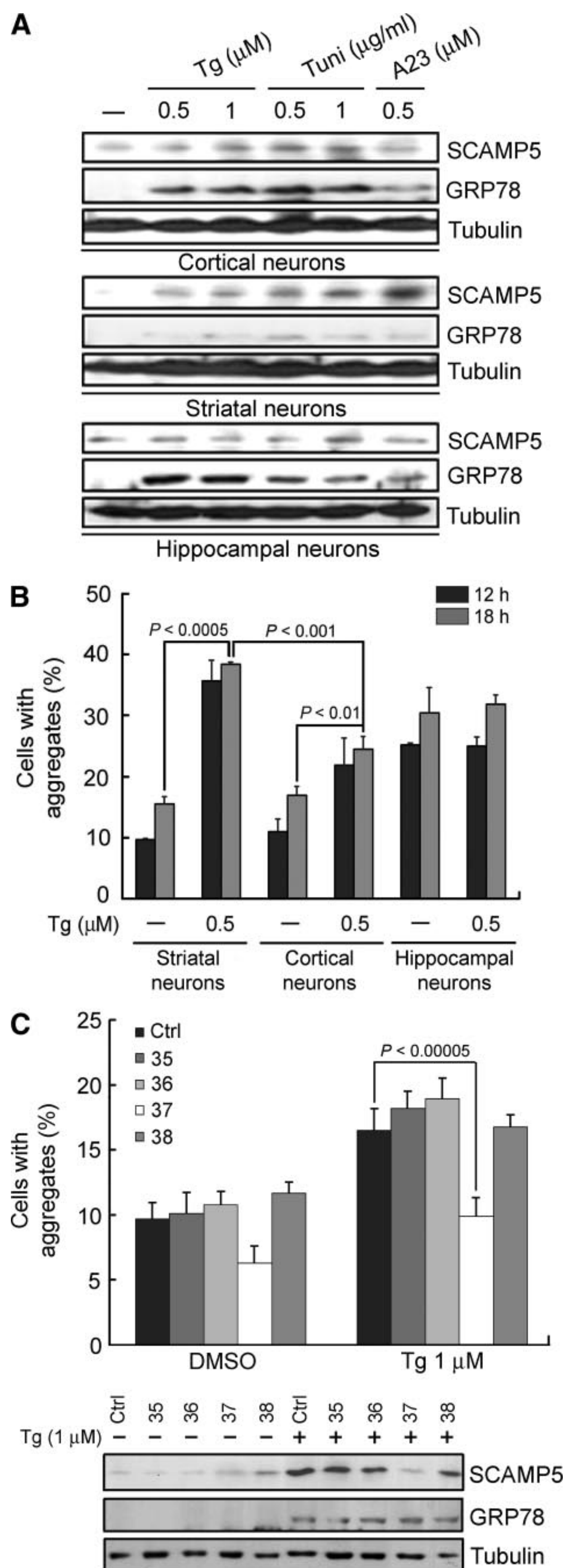

FIGURE 2. **ER stress increases the aggregation of mtHTT (HTTex120Q-GFP) via up-regulation of SCAMP5.** *A*, induction of SCAMP5 by ER stress. Primary rat striatal, cortical, and hippocampal neurons were cultured from embryonic day 16, maintained *in vitro* for 3 days, and then left untreated or exposed to A23187 (*A23*), tunicamycin (*Tuni*), or thapsigargin (*Tg*). After 24 h, cells were harvested and examined with Western blotting using anti-SCAMP5 and anti-GRP78 antibodies. *B*, increased aggregation of HTTex120Q-GFP by ER stress. Primary rat striatal, cortical, and hippocampal neurons were transfected with HTTex120Q-GFP for 18 h and then left untreated or exposed to 0.5 μM thapsigargin for the indicated times. Cells were then examined for the aggregation of HTTex120Q-GFP under a fluorescence microscope. *Bars* represent mean values ± S.D. ($n = 3$). *C*, reduction of ER stress-induced aggregation of HTTex120Q-GFP in SCAMP5 knockdown cells. SH-SY5Y cells were transfected with control (*Ctrl*; pLKO.1) or SCAMP5 short hairpin RNA (TRCN0000105535∼TRCN0000105538) and selected with puromycin. Mixed populations of cells were transfected with HTTex120Q-GFP for 18 h, incubated with thapsigargin (Tg) for 18 h, and examined for the aggregation of HTTex120Q-GFP under a fluorescence microscope (*upper panel*). After that, cells (*Ctrl*; 35∼38) were examined for the expression level of SCAMP5 with Western blot analysis (*lower panel*). *Bars* represent mean values ± S.D. from at least three independent experiments. *DMSO*, dimethyl sulfoxide.

resuspended in a volume of PBS equal to the volume of supernatant.

Post-mortem Brain Samples—Striatum samples from five HD patients (grade 3) and three age-matched controls were from Boston University School of Medicine (Bedford, MA).

R6/2 Mice—R6/2 mice, which are transgenic for exon 1 of the human HD gene, carrying about 115–150 glutamine repeats (The Jackson Laboratory), and their wild-type littermates were used at 9-week age ranges.

Generation of Stable Cells—SH-SY5Y cells were transfected with SCAMP5 short hairpin RNA (OpenBiosystem: TRCN0000105535∼TRCN0000105538) for 24 h and then incubated with 3 μg/ml puromycin (Invitrogen) for 5 days for the generation of mixed cell populations (SH-SY5Y/ctrl and SH-SY5Y/35–38). HTT 103Q-GFP/PC12 cells (Htt14A2.5/ecdysone-inducible PC12 cells) were kindly provided by Dr. L. M. Thompson (University of California).

Transferrin Uptake Assay—Cells were grown on a coverslip coated with poly-L-lysine and starved for 30 min. Thereafter, cells were incubated with Alexa 594-labeled transferrin (25 μg/ml) (Molecular Probes) for 15 min, washed three times with ice-cold PBS on ice, and then fixed with 4% paraformaldehyde for observation under a confocal microscope.

Cell Death Assay—Cells were stained with 5 μM ethidium homodimer (Molecular Probes) for 10 min and then examined under fluorescence microscope.

RESULTS

Functional Isolation of SCAMP5 as an Enhancer of mtHTT Aggregation—To find out new regulators of polyQ track protein aggregation, we screened 4,200 human full-length cDNAs in the expression vector with cell-based assays. We examined overexpression effects of each clone on the aggregation of GFP-fused polyglutamine ($n = 110$) (poly110Q-GFP) or GFP-fused segment of HTT exon 1 containing expanded polyglutamine ($n = 120$) (HTTex120Q-GFP) (26). When these polyQ track constructs were expressed in cells, GFP allowed the detection of expression, aggregation, and subcellular localization of the chimera under a fluorescence microscope. During the screening, transfection efficiency, which was normalized by co-transfection with monomeric RFP (mRFP), was relatively even for the clones, and GFP-positive dots were counted as aggregates of the polyQ track protein. We confirmed HTTex120Q-GFP dots as

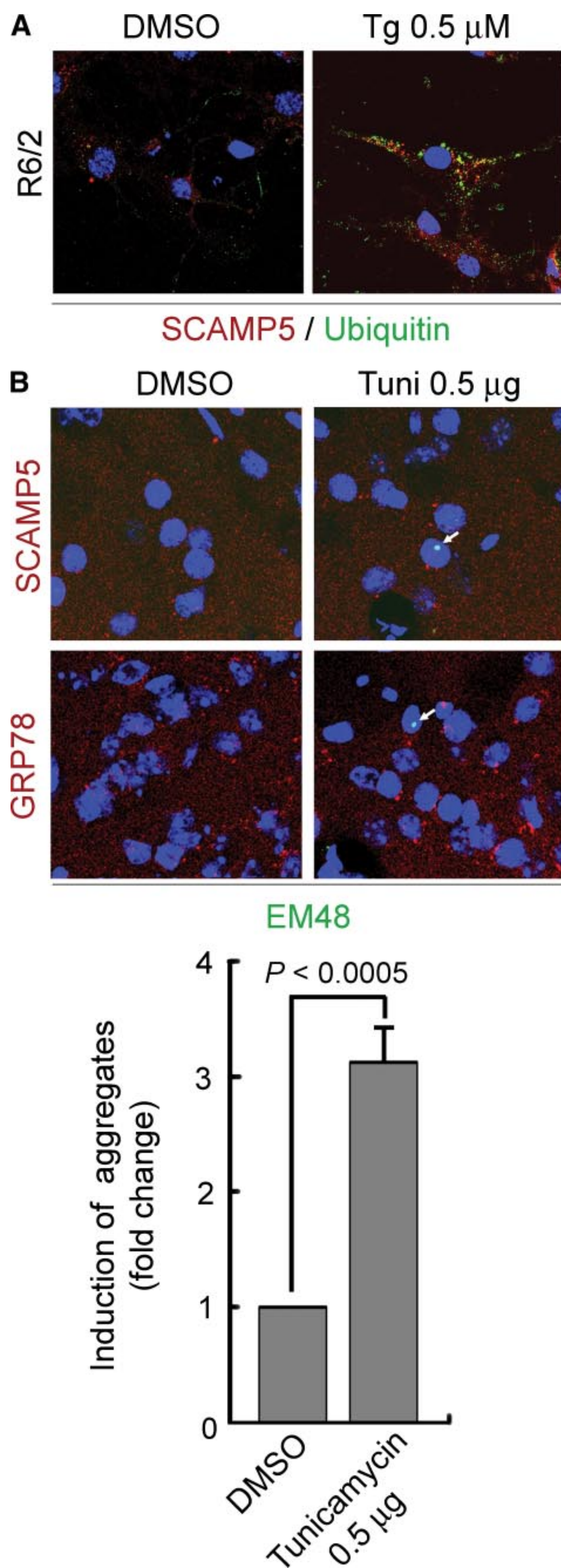

FIGURE 3. **ER stress increases mtHTT aggregation in the primary striatal cells and striatal tissues of R6/2 mice.** *A*, thapsigargin (*Tg*) induces ubiquitin-positive aggregation in the primary striatal neurons of R6/2 mice. Primary striatal neurons were cultured from newborn R6/2 mice at day 2 and maintained *in vitro* for 3 days. Thereafter, cells were incubated with dimethyl sulfoxide (*DMSO*) or 0.5 μM thapsigargin for 24 h and co-stained with anti-ubiquitin (*green*) and anti-SCAMP5 (*red*) antibodies. Nuclei were stained by Hoechst 33342 (*blue*). *B*, tunicamycin (*Tuni*) increases the aggregation of mtHTT in the striatal tissues of R6/2 mice. The striata of R6/2 mice were directly injected with dimethyl sulfoxide (left striatum) or tunicamycin (0.5 μg, right striatum) at 60 days of age. Three days after the injection, frozen sections of brain tissues were co-stained with anti-mutant huntingtin antibody (EM48) (*green*; *arrow*) and either anti-SCAMP5 (*red*) or anti-GRP78 (*red*) antibody (*upper panel*). Nuclei were stained by Hoechst 33342 (*blue*). EM48-positive aggregates in cells were counted under a confocal microscope, and its relative ratio is represented as -fold change (*lower panel*).

protein aggregates with immunostaining assays using anti-ubiquitin antibody and detergent-resistant assays (supplemental Fig. S1, *B* and *C*). From the secondary screening, we isolated 24 putative positive clones that regulate the aggregation of polyQ track protein in cell culture (data not shown). In particular, SCAMP5 exhibited strong effects on the aggregation of HTTex120Q-GFP in a dose-dependent manner during secondary screening (supplemental Fig. S1*A*). SCAMP5 is believed to be a vesicle membrane protein with unclear functions (30) and shows brain-specific expression. Thus, a role for SCAMP5 was assessed in detail.

Ectopic expression of SCAMP5 increased the number of cells showing HTTex120Q-GFP aggregates in the primary striatal neurons (Fig. 1*A*), SH-SY5Y neuronal cells (supplemental Fig. S1*A*) and HEK293F non-neuronal cells (supplemental Fig. S1*D*), but did not enhance the aggregation of huntingtin exon 1 containing a normal length of polyQ ($n = 18$) (Fig. 1*B*). By employing ecdysone-inducible long term expression of the GFP-tagged mutant huntingtin fragment encoding exon 1 with 103 glutamines (HTT 103Q-GFP) in PC12 neuronal cells (27), we also observed that the number of cells with HTT 103Q-GFP aggregates was increased 4-fold by the expression of SCAMP5 (Fig. 1*C*). This SCAMP5-mediated aggregation of HTTex120Q-GFP was increased in a dose-dependent manner in AF5 striatal neuronal cells (28) (data not shown).

SCAMP5 Is Increased in Striatum of HD Patients and in Cultured Cells by ER Stress—Next, we determined the expression level of SCAMP5 in the striatum of HD patients. SCAMP5 was markedly increased in the striatum of HD patients (grade 3) compared with age-matched controls (Fig. 1*D*). In addition, the level of SCAMP5 was also increased at day 6 in PC12 neuronal cells by ecdysone-inducible expression of HTT 103Q-GFP (Fig. 1*E*). Interestingly, an ER stress marker, CHOP, was induced by HTT 103Q-GFP (Fig. 1*E*), which prompted us to examine the interaction between ER stress and polyQ track protein aggregation.

Notably, we found that SCAMP5 was highly induced in the primary striatal neurons in response to ER stress triggered by tunicamycin (an inhibitor of protein glycosylation), thapsigargin (an ER Ca^{2+}-ATPase inhibitor), or A23187 (a Ca^{2+} ionophore) but not in hippocampal neurons (Fig. 2*A*). We also found that the expression of SCAMP5 was induced in SH-SY5Y and AF5 neuronal cell lines exposed to ER stress (data not shown). From reverse transcription (RT)-PCR analysis, we observed that the mRNA level of SCAMP5 was not significantly changed by thapsigargin and tunicamycin (data not shown),

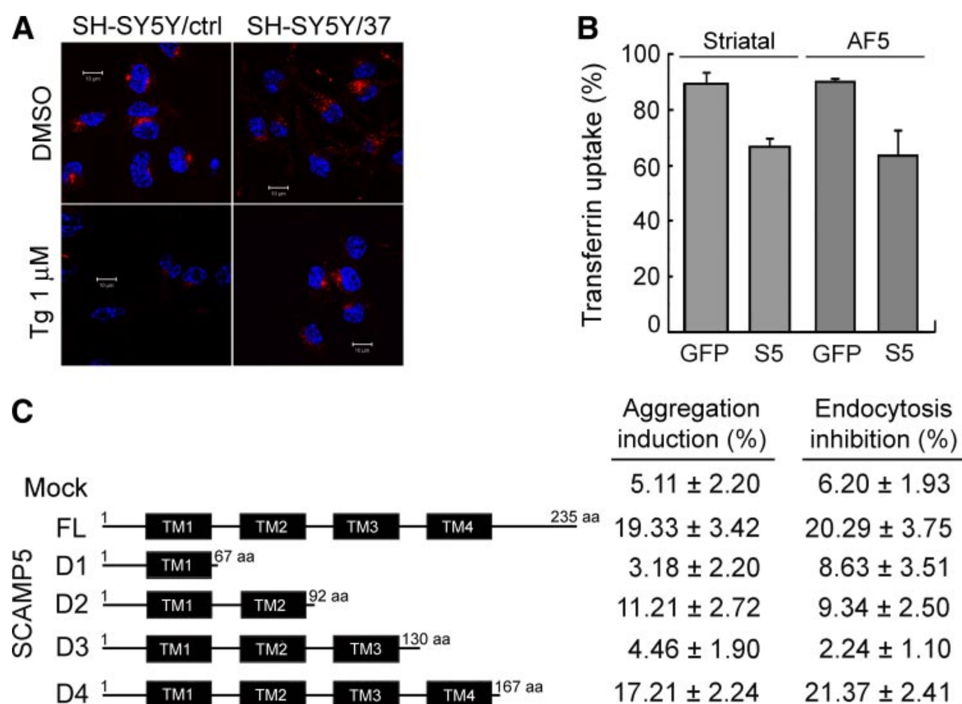

FIGURE 4. **SCAMP5 induces the aggregation of mtHTT (HTTex120Q-GFP) via endocytosis inhibition.** *A*, ER stress impairs receptor-mediated endocytosis via SCAMP5. SH-SH5Y/ctrl and SH-SH5Y/37 cells were exposed to thapsigargin (*Tg*) for 24 h and then incubated with 25 μg/ml transferrin-Alexa 594 (*red*) and Hoechst 33342 (*blue*). Cells were observed under a confocal microscope. *DMSO*, dimethyl sulfoxide. *B*, ectopic expression of SCAMP5 suppresses endocytosis in the primary striatal neurons. Primary rat striatal neurons were transfected with either GFP or SCAMP5-GFP (*S5*) for 24 h. After incubation with transferrin/Alexa 594, cells were observed under confocal microscope. The percentage of transferrin uptake (mean values ± S.D.; $n = 3$) was determined in the primary striatal neurons and AF5 cells using captured images with a confocal microscope. *C*, shown are the effects of SCAMP5 deletion mutants on HTTex120Q-GFP aggregation and endocytosis inhibition. Shown is a schematic diagram of SCAMP5 (*FL*) and its deletion (*D*) (*left panel*). SH-SY5Y cells were transfected with GFP (*Mock*), SCAMP5-GFP (*FL*), or its deletion mutants (D1–D4) for 24 h and transferrin uptake assay was then performed as in *A*. For aggregation assays, SH-SY5Y cells were co-transfected with HTTex120Q-GFP and either SCAMP5-HA or its deletion mutants for 24 h. The results are summarized with mean values ± S.D. from at least three independent experiments (*right panel*).

suggesting that the increase of SCAMP5 may be regulated at the post-transcriptional level.

ER Stress Induces the Aggregation of mtHTT via SCAMP5—We then examined the effects of ER stress on the aggregation of mtHTT. Exposure to thapsigargin highly increased the aggregation of HTTex120Q-GFP in the primary striatal neurons (3.5-fold) (Fig. 2*B*) and in the NeuN-positive primary striatal neurons (data not shown) but not in the hippocampal neurons. Similarly, thapsigargin significantly increased both SCAMP5 expression and HTTex120Q-GFP aggregation in SH-SY5Y cells (Fig. 2*C*).

Next, we generated stable SH-SY5Y cell lines showing the reduced expression of SCAMP5 (SH-SY5Y/37) with short hairpin RNA (TRCN0000105537) (Fig. 2*C*, *lower panel*). The expression of SCAMP5 was not increased in SH-SY5Y/37 cells in response to thapsigargin compared with control cells (SH-SY5Y/ctrl). (Fig. 2*C*, *lower panel*). We also found that thapsigargin-induced aggregation of HTTex120Q-GFP was not increased in SH-SY5Y/37 cells (Fig. 2*C*, *upper panel*). These observations suggest that the expression level of SCAMP5 is critical for the regulation of ER stress-induced aggregation of HTTex120Q-GFP.

In addition, thapsigargin treatment increased both SCAMP5 expression and ubiquitin-positive cytosolic aggregates in the primary striatal neurons cultured from newborn R6/2 HD model mice (29) (Fig. 3*A*). Moreover, direct injection of tunicamycin into the striatum of R6/2 mice increased EM48-positive and nuclear aggregation of mtHTT (*arrows*) as well as the expression of SCAMP5 and GRP78 in the same cells of striatal sections (Fig. 3*B*). In parallel, intraperitoneal injection of tunicamycin enhanced nuclear aggregation of mtHTT in the cortex of N171-82Q mice (data not shown).

SCAMP5 Induces mtHTT Aggregation via Endocytosis Inhibition—SCAMP5 shows significant amino acid sequence identity (45–57%) with other members of the SCAMP family. The observation that the N-terminal deletion mutant of SCAMP1 has a structural similarity with SCAMP5 and exhibits the inhibitory effect on receptor-mediated endocytosis led us to examine the effect of SCAMP5 on endocytosis (31). We found that thapsigargin treatment inhibited transferrin uptake in SH-SY5Y cells (Fig. 4*A*). However, this inhibition of transferrin uptake was alleviated in SH-SY5Y/37 cells, which express reduced amounts of SCAMP5. Likewise, thapsigargin inhibited transferrin uptake in the primary striatal neurons (supplemental Fig. S2*A*). In addition, ectopic expression of SCAMP5 also suppressed transferrin uptake to 70% of control cells in the primary striatal neurons and AF5 cells (Fig. 4*B*) as well as in SH-SY5Y cells (data not shown). These results suggest that the increased expression of SCAMP5 impairs receptor-mediated endocytosis.

To define the domain(s) responsible for endocytosis inhibition and mtHTT aggregation, several deletion mutants of SCAMP5 (SCAMP5-D) were generated (Fig. 4*C*, *left panel*). Like the wild type, the SCAMP5-D4 mutant lacking the C terminus effectively inhibited endocytosis and mtHTT aggregation. However, further deletion from the C terminus, such as SCAMP5-D3 mutant losing transmembrane domain 4, abrogated the ability of SCAMP5 to inhibit endocytosis and to induce mtHTT aggregation, whereas deletion of transmembrane domains 3 and 4 seems to partially recover these activities. These results show a good correlation between the ability of SCAMP5 to inhibit endocytosis and to increase mtHTT aggregation, and most regions of SCAMP5, except the C-terminal tail region spanning residues 167–235, are required for the aggregation of mtHTT (Fig. 4*C*, *right panel*). Also, the association of endocytosis inhibition with the increased aggregation of mtHTT was evident in our analysis employing the endocytosis

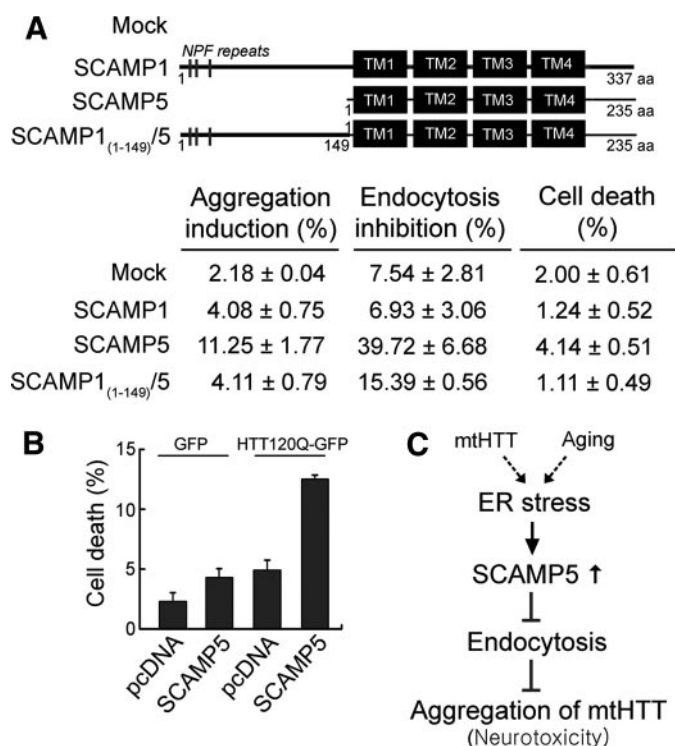

FIGURE 5. **Ectopic expression of SCAMP5 enhances the neurotoxicity of mtHTT.** *A*, shown is the effect of the SCAMP5 chimera (SCAMP1-(1–149)/5) on HTTex120Q-GFP aggregation, endocytosis, and cell death. A schematic diagram of the SCAMP1-(1–149)/5 chimera is shown (*upper panel*). SH-SY5Y cells were transfected with the SCAMP1, SCAMP5, or SCAMP1-(1–149)/5 chimera with HTTex120Q-GFP for 24 h and then analyzed for mtHTT aggregation under a fluorescence microscope. For a transferrin uptake assay, SH-SY5Y cells were transfected with the indicated construct for 24 h and then assessed for endocytosis as described in the legend of Fig. 4. Cells were then captured into images with the same exposure time under a confocal microscope, and the numbers of transferrin-positive cells on the images were counted. The results are summarized with mean values ± S.D. from at least three independent experiments (*lower panel*). For cell death assay, SH-SY5Y cells were co-transfected with GFP and the indicated plasmid for 24 h, stained with ethidium homodimer, and examined under a microscope. *B*, SCAMP5 enhances the neurotoxicity of mtHTT. SH-SY5Y cells were transfected for 24 h with GFP or HTTex120Q-GFP together with pcDNA or SCAMP5 as indicated and examined under the microscope after staining with ethidium homodimer. *C*, shown is the proposed role of SCAMP5 in the regulation of mtHTT aggregation.

inhibitor. Dynamin K44A mutant, a dominant-negative mutant of endocytosis induced SDS-insoluble aggregates of mtHTT (supplemental Fig. S2*B*). These data support our hypothesis that the inhibition of endocytosis pathway by SCAMP5 may be critical to regulate the aggregation of mtHTT.

SCAMP1 has multiple NPF (asparagine-proline-phenylalanine) repeats, which are known as binding partners of the EPS15 homology domain of intersectin in its N terminus. To obtain further insight into a role of SCAMP5 in endocytosis, a chimera (SCAMP1-(1–149)/5) was generated by fusing the N terminus of the SCAMP1-(1–149)-containing NPF repeats with SCAMP5 (Fig. 5*A*, *upper panel*). Compared with SCAMP5, ectopic expression of the SCAMP1-(1–149)/5 chimera was much less able to induce mtHTT aggregation and to inhibit endocytosis (Fig. 5*A*, *lower panel*). Also, addition of the N terminus of SCAMP1 did not change the subcellular localization of SCAMP5 (supplemental Fig. S2*C*). SCAMP5 was reported to be located in synaptic vesicles (30) and located in the plasma membrane (supplemental Fig. S2*C*). These results support our proposal that the SCAMP5-mediated inhibition of endocytosis is linked to mtHTT aggregation and suggest that SCAMP5 may function as a negative regulator of endocytosis. In addition, ectopic expression of SCAMP5 was not toxic to cells (Fig. 5, *A* and *B*) but enhanced the toxicity of mtHTT when coexpressed with mtHTT in neuronal cells (Fig. 5*B*), suggesting that the SCAMP5-mediated increase of mtHTT aggregation may be linked to the neurotoxic activity of mtHTT.

DISCUSSION

Despite the discrepancy between polyQ track protein aggregation and neurodegeneration (33, 34), cellular aggregation of expanded polyQ track protein is considered as a major pathogenic factor in polyglutamine disease (2, 9, 29, 35). Genome-wide functional screenings have been performed by several groups. Hundreds of huntingtin-interacting proteins were isolated using pulldown and yeast two-hybrid assays (36). Also, modifiers of ataxin-3 neurodegeneration and polyglutamine aggregation were screened with thousands of fly (37) and *Caenorhabditis elegans* mutant lines (38). We isolated 24 enhancers of polyQ track protein aggregation using cell-based assay. Compared with the previous screenings, many genes functioning in the protein and vesicle transport process were isolated from our assays, and the aggregation enhancers identified here did not much overlap with those isolated by the other group. Despite some limitation of the overexpression and relative expression levels of each clone, our screening using cDNA overexpression can be an effective way to isolate new modifiers of polyglutamine disease.

The three SCAMP proteins (SCAMP1–3) share a common domain structure composed of a cytoplasmic N-terminal domain with multiple NPF repeats, four highly conserved transmembrane regions, and a short cytoplasmic C-terminal tail. Interestingly, SCAMP4 and SCAMP5 lack the N-terminal NPF repeats that are highly conserved in all other SCAMP proteins (30). Among them, SCAMP5 was most potent in increasing mtHTT aggregation (data not shown). SCAMP5 is apparently induced in AF5 and SH-SY5Y neuronal cells by ER stress. ER stress-induced up-regulation of SCAMP5 was also evident in the striatum and cortex tissues and in cultured primary striatal and cortical cells.

Interestingly, polyQ track protein is able to induce ER stress and to increase the expression of SCAMP5. Thus, ER stress, which was also observed by other groups to be generated by mtHTT (17–19), may be mediated by SCAMP5. This regulation may form a positive feedback loop to accelerate the aggregation of polyQ track protein. Furthermore, treatment with tumor necrosis factor-α and oxidative stress, such as H_2O_2, was also able to increase the expression of SCAMP5 in cultured cells (data not shown). Thus, we hypothesize that the increase of SCAMP5 in the brains of HD patients may be caused by pathogenic stresses, including ER stress, inflammation, and oxidative stress as well as genetic alteration in huntingtin and may contribute to the accumulation of polyQ track proteins for neurotoxicity (Fig. 5*C*).

Autophagy is getting more attention as a clearance pathway of polyQ track protein. Inhibition of autophagy increases aggre-

gate formation of mtHTT (23, 25), while activation of autophagy by rapamycin, an inhibitor of mTOR, rescues huntingtin-induced degeneration in flies (24). The autophagic process initiates with the formation of autophagic vesicles (23, 24, 40), and the degradation of such autophagic vesicles is followed by fusion with lysosomes (40). Recent papers show that the perturbation of endocytosis causes malfunction of autophagic clearance (41–43), suggesting that endocytosis may be tightly associated with the autophagic process. Thus, we suggest that the inhibition of endocytosis by SCAMP5 may reduce lysosomal degradation of polyQ track protein by autophagic clearance, resulting in the accumulation of autophagosomes.

Furthermore, we found a large portion of endocytotic proteins (33%) and signal transduction proteins (33%) to be effective modulators of mtHTT aggregation such as RAB4A (member of the Ras oncogene family), RABGEF1 (RAB guanine nucleotide exchange factor 1), SEC23B (Sec23 homolog B), SEC24D (SEC24-related gene family, member D), SMAP1 (stromal membrane-associated protein 1), and Slp2 (synaptotagmin-like protein 2) from our cell-based assay. Thus, vesicle transport, including endocytosis, seems to be required for the maintenance of lysosomal biogenesis (46) and for autophagic clearance (44). Although molecular detail on how the increased expression of SCAMP5 regulates endocytosis remains to be further addressed, we propose that SCAMP5-mediated interference of endocytosis may affect lysosomal activity, which eventually impairs the removal process of mtHTT aggregates. Taken together, these results elucidate that SCAMP5 is a key regulatory molecule linking ER stress to the aggregation of polyQ track protein via endocytosis modulation.

Acknowledgments—We thank Dr. L. M. Thompson (University of California) for Htt14A2.5/ecdysone-inducible PC12 cells; Dr. A. Dautry-Varsat (Institute Pasteur, Paris, France) for EPS15-Δ95/295-GFP, Dynamin K44A-GFP, and synaptotagmin VII-GFP (Syt VII); and Dr. S. Chang (Gwangju Institute of Science and Technology, Gwangju, Korea) for technical assistance.

REFERENCES

1. Gusella, J. F., and MacDonald, M. E. (2000) *Nat. Rev. Neurosci.* **1,** 109–115
2. Zoghbi, H. Y., and Orr, H. T. (2007) *Annu. Rev. Neurosci.* **30,** 575–621
3. Soto, C. (2003) *Nat. Rev. Neurosci.* **4,** 49–60
4. Tam, S., Geller, R., Spiess, C., and Frydman, J. (2006) *Nat. Cell Biol.* **8,** 1155–1162
5. Gokhale, K. C., Newnam, G. P., Sherman, M. Y., and Chernoff, Y. O. (2005) *J. Biol. Chem.* **280,** 22809–22818
6. Behrends, C., Langer, C. A., Boteva, R., Böttcher, U. M., Stemp, M. J., Schaffar, G., Rao, B. V., Giese, A., Kretzschmar, H., Siegers, K., and Hartl, F. U. (2006) *Mol. Cell* **23,** 887–897
7. Muchowski, P. J. (2002) *Neuron* **35,** 9–12
8. Rubinsztein, D. C. (2002) *Trends Genet.* **18,** 202–209
9. Sánchez, I., Mahlke, C., and Yuan, J. (2003) *Nature* **421,** 373–379
10. Høyer-Hansen, M., and Jäättelä, M. (2007) *Cell Death Differ.* **14,** 1576–1582
11. Yoshida, H. (2007) *FEBS J.* **274,** 630–658
12. Uehara, T., Nakamura, T., Yao, D., Shi, Z. Q., Gu, Z., Ma, Y., Masliah, E., Nomura, Y., and Lipton, S. A. (2006) *Nature* **441,** 513–517
13. Ozcan, U., Yilmaz, E., Ozcan, L., Furuhashi, M., Vaillancourt, E., Smith, R. O., Görgün, C. Z., and Hotamisligil, G. S. (2006) *Science* **313,** 1137–1140
14. Zhang, K., Shen, X., Wu, J., Sakaki, K., Saunders, T., Rutkowski, D. T., Back, S. H., and Kaufman, R. J. (2006) *Cell* **124,** 587–599
15. Kitao, Y., Imai, Y., Ozawa, K., Kataoka, A., Ikeda, T., Soda, M., Nakimawa, K., Kiyama, H., Stern, D. M., Hori, O., Wakamatsu, K., Ito, S., Itohara, S., Takahashi, R., and Ogawa, S. (2007) *Hum. Mol. Genet.* **16,** 50–60
16. Song, S., Lee, H., Kam, T. I., Tai, M. L., Lee, J. Y., Noh, J. Y., Shim, S. M., Seo, S. J., Kong, Y. Y., Nakagawa, T., Chung, C. W., Choi, D. Y., Oubrahim, H., and Jung, Y. K. (2008) *J. Cell Biol.* **182,** 675–684
17. Nishitoh, H., Matsuzawa, A., Tobiume, K., Saegusa, K., Takeda, K., Inoue, K., Hori, S., Kakizuka, A., and Ichijo, H. (2002) *Genes Dev.* **16,** 1345–1355
18. Kouroku, Y., Fujita, E., Jimbo, A., Kikuchi, T., Yamagata, T., Momoi, M. Y., Kominami, E., Kuida, K., Sakamaki, K., Yonehara, S., and Momoi, T. (2002) *Hum. Mol. Genet.* **11,** 1505–1515
19. Reijonen, S., Putkonen, N., Nørremølle, A., Lindholm, D., and Korhonen, L. (2008) *Exp. Cell Res.* **314,** 950–960
20. Bence, N. F., Sampat, R. M., and Kopito, R. R. (2001) *Science* **292,** 1552–1555
21. Bennett, E. J., Bence, N. F., Jayakumar, R., and Kopito, R. R. (2005) *Mol. Cell* **17,** 351–365
22. Bennett, E. J., Shaler, T. A., Woodman, B., Ryu, K. Y., Zaitseva, T. S., Becker, C. H., Bates, G. P., Schulman, H., and Kopito, R. R. (2007) *Nature* **448,** 704–708
23. Ravikumar, B., Duden, R., and Rubinsztein, D. C. (2002) *Hum. Mol. Genet.* **11,** 1107–1117
24. Ravikumar, B., Vacher, C., Berger, Z., Davies, J. E., Luo, S., Oroz, L. G., Scaravilli, F., Easton, D. F., Duden, R., O'Kane, C. J., and Rubinsztein, D. C. (2004) *Nat. Genet.* **36,** 585–595
25. Williams, A., Jahreissa, L., Sarkara, S., Saikia, S., Menziesa, M. F., Ravikumara, B., and Rubinsztein, D. C. (2006) *Curr. Top. Dev. Biol.* **76,** 89–101
26. Kim, M., Lee, H. S., LaForet, G., McIntyre, C., Martin, E. J., Chang, P., Kim, T. W., Williams, M., Reddy, P. H., Tagle, D., Boyce, F. M., Won, L., Heller, A., Aronin, N., and DiFiglia, M. (1999) *J. Neurosci.* **19,** 964–973
27. Apostol, B. L., Kazantsev, A., Raffioni, S., Illes, K., Pallos, J., Bodai, L., Slepko, N., Bear, J. E., Gertler, F. B., Hersch, S., Housman, D. E., Marsh, J. L., and Thompson, L. M. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 5950–5955
28. Truckenmiller, M. E., Vawter, M. P., Zhang, P., Conejero-Goldberg, C., Dillon-Carter, O., Morales, N., Cheadle, C., Becker, K. G., and Freed, W. J. (2002) *Exp. Neurol.* **175,** 318–337
29. Mangiarini, L., Sathasivam, K., Seller, M., Cozens, B., Harper, A., Hetherington, C., Lawton, M., Trottier, Y., Lehrach, H., Davies, S. W., and Bates, G. P. (1996) *Cell* **87,** 493–506
30. Fernández-Chacón, R., and Südhof, T. C. (2000) *J. Neurosci.* **20,** 1941–1950
31. Fernández-Chacón, R., Achiriloaie, M., Janz, R., Albanesi, J. P., and Südhof, T. C. (2000) *J. Biol. Chem.* **275,** 12752–12756
32. Chang, J. W., Choi, H., Kim, H. J., Jo, D. G., Jeon, Y. J., Noh, J. Y., Park, W. J., and Jung, Y. K. (2007) *Hum. Mol. Genet.* **16,** 317–326
33. Saudou, F., Finkbeiner, S., Devys, D., and Greenberg, M. E. (1998) *Cell* **95,** 55–66
34. Arrasate, M., Mitra, S., Schweitzer, E. S., Segal, M. R., and Finkbeiner, S. (2004) *Nature* **431,** 805–810
35. Davies, S. W., Turmaine, M., Cozens, B. A., DiFiglia, M., Sharp, A. H., Ross, C. A., Scherzinger, E., Wanker, E. E., Mangiarini, L., and Bates, G. P. (1997) *Cell* **90,** 537–548
36. Kaltenbach, L. S., Romero, E., Becklin, R. R., Chettier, R., Bell, R., Phansalkar, A., Strand, A., Torcassi, C., Savage, J., Hurlburt, A., Cha, G. H., Ukani, L., Chepanoske, C. L., Zhen, Y., Sahasrabudhe, S., Olson, J., Kurschner, C., Ellerby, L. M., Peltier, J. M., Botas, J., and Hughes, R. E. (2007) *PLoS Genet.* **3,** e82
37. Bilen, J., and Bonini, N. M. (2007) *PLoS Genet.* **10,** 1950–1964
38. Nollen, E. A., Garcia, S. M., van Haaften, G., Kim, S., Chavez, A., Morimoto, R. I., and Plasterk, R. H. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 6403–6408
39. Song, S., Kim, S. Y., Hong, Y. M., Jo, D. G., Lee, J. Y., Shim, S. M., Chung, C. W., Seo, S. J., Yoo, Y. J., Koh, J. Y., Lee, M. C., Yates, A. J., Ichijo, H., and Jung, Y. K. (2003) *Mol. Cell* **12,** 553–563
40. Ravikumar, B., Acevedo-Arozena, A., Imarisio, S., Berger, Z., Vacher, C.,

O'Kane, C. J., Brown, S. D., and Rubinsztein, D. C. (2005) *Nat. Genet.* **37,** 771–776
41. Lee, J. A., Beigneux, A., Ahmad, S. T., Young, S. G., and Gao, F. B. (2007) *Curr. Biol.* **17,** 1561–1567
42. Rusten, T. E., Vaccari, T., Lindmo, K., Rodahl, L. M., Nezis, I. P., Sem-Jacobsen, C., Wendler, F., Vincent, J. P., Brech, A., Bilder, D., and Stenmark, H. (2007) *Curr. Biol.* **17,** 1817–1825
43. Filimonenko, M., Stuffers, S., Raiborg, C., Yamamoto, A., Malerød, L., Fisher, E. M., Isaacs, A., Brech, A., Stenmark, H., and Simonsen, A. (2007) *J. Cell Biol.* **179,** 485–500
44. Nielsen, R., Courtoy, P. J., Jacobsen, C., Dom, G., Lima, W. R., Jadot, M., Willnow, T. E., Devuyst, O., and Christensen, E. I. (2007) *Proc. Natl. Acad. Sci. U. S. A.* **104,** 5407–5412
45. Suzuki, Y., Ishihara, D., Sasaki, M., Nakagawa, H., Hata, H., Tsunoda, T., Watanabe, M., Komatsu, T., Ota, T., Isogai, T., Suyama, A., and Sugano, S. (2000) *Genomics* **64,** 286–297
46. Hennig, K. M., Colombani, J., and Neufeld T. P. (2006) *J. Cell Biol.* **173,** 963–974