

Rapamycin Inhibits Polyglutamine Aggregation Independently of Autophagy by Reducing Protein Synthesis^S

Matthew A. King, Sarah Hands, Farida Hafiz, Noboru Mizushima, Aviva M. Tolkovsky, and Andreas Wytenbach

Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom (M.A.K., F.H., A.M.T., A.W.); Department of Physiology and Cell Biology, Tokyo Medical and Dental University, Tokyo, Japan (N.M.); and School of Biological Sciences, Neuroscience Group, University of Southampton, Southampton, United Kingdom (S.H., A.W.)

Received November 12, 2007; accepted December 31, 2007

ABSTRACT

Accumulation of misfolded proteins and protein assemblies is associated with neuronal dysfunction and death in several neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's disease (HD). It is therefore critical to understand the molecular mechanisms of drugs that act on pathways that modulate misfolding and/or aggregation. It is noteworthy that the mammalian target of rapamycin inhibitor rapamycin or its analogs have been proposed as promising therapeutic compounds clearing toxic protein assemblies in these diseases via activation of autophagy. However, using a cellular model of HD, we found that rapamycin significantly decreased aggregation-prone polyglutamine (polyQ) and expanded huntingtin and its inclusion bodies (IB) in both autophagy-proficient and autophagy-deficient cells (by genetic knockout of the *atg5* gene in mouse embryonic fibro-

blasts). This result suggests that rapamycin modulates the levels of misfolded polyQ proteins via pathways other than autophagy. We show that rapamycin reduces the amount of soluble polyQ protein via a modest inhibition of protein synthesis that in turn significantly reduces the formation of insoluble polyQ protein and IB formation. Hence, a modest reduction in huntingtin synthesis by rapamycin may lead to a substantial decrease in the probability of reaching the critical concentration required for a nucleation event and subsequent toxic polyQ aggregation. Thus, in addition to its beneficial effect proposed previously of reducing polyQ aggregation/toxicity via autophagic pathways, rapamycin may alleviate polyQ disease pathology via its effect on global protein synthesis. This finding may have important therapeutic implications.

The polyglutamine/CAG disorders comprise a group of neurodegenerative diseases that are associated with polyglutamine (polyQ) expansion mutations in the respective disease genes that are otherwise unrelated (Cummings and Zoghbi, 2000). Abnormally long polyQ stretches cause proteins to misfold and produce intracellular protein aggregates. It is believed that polyQ aggregation follows a stochastic nucleation-dependent process that initiates oligomerization, amyloid-like fibril formation, and the production of structures called inclusion bodies (IBs) (Perutz and Windle, 2001).

Because polyQ misfolding/aggregation is associated with cellular toxicity (Ross and Poirier, 2004), it is crucial to understand the cellular mechanisms that control misfolding/aggregation with a view to the development of drugs that modify these pathways and alleviate disease.

The accumulation of intracellular IBs points to the inability of cells to dispose of mutant polyQ proteins using chaperone-assisted refolding (Muchowski and Wacker, 2005) and proteasome-mediated degradation (Jana and Nukina, 2003). Deciphering the mechanisms of degradation and clearance of polyQ-expanded proteins and how such mechanisms might be targeted using drugs is a major focus of current research. Macroautophagy (here referred to as autophagy) is a process alternative to that of proteasomal degradation by which some long-lived proteins and organelles are cleared (Shintani and Klionsky, 2004). Autophagy may be responsible for clearing polyQ-expanded proteins and their assemblies (Rubinshtein,

This study was supported by the Wellcome Trust before October 2006 (to M.A.K., A.M.T.), the Hereditary Disease Foundation (to F.H.), and the Medical Research Council (to A.W., S.H.).

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.107.043398.

^S The online version of this article (available at <http://molpharm.aspetjournals.org>) contains supplemental material.

ABBREVIATIONS: polyQ, polyglutamine; CHX, cycloheximide; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; Htt, huntingtin protein; Ex1Htt, huntingtin exon 1; HD, Huntington's disease; IB, inclusion body; MEF, mouse embryonic fibroblast; mRFP, monomeric red fluorescent protein; mTOR, mammalian target of rapamycin; BafA1, bafilomycin A1; ANOVA, analysis of variance; PAGE, polyacrylamide gel electrophoresis; Rap, rapamycin; FK506, tacrolimus; CCI-779, temsirolimus; ERK, extracellular signal-regulated kinase; tERK, total extracellular signal-regulated kinase.

2006). Clearance by autophagy occurs by sequestration of the target organelle/protein into double-membrane structures called autophagosomes that fuse with endo/lysosomes and discharge their contents, which are subsequently degraded. The mammalian homolog of Atg8 MAP-LC3 (LC3) is a key mediator of autophagy: after LC3 is C-terminally cleaved (LC3 I), phosphatidylethanolamine is added to the C-terminal glycine by the Atg5/12 complex, generating LC3 II bound to the nascent autophagosomal membrane (Tanida et al., 2004). Because the Atg5/12 complex catalytically activates the lipidation of LC3, trace amounts of Atg5 can support substantial autophagy, whereas Atg5 knockout cells are totally deficient in autophagy (Hosokawa et al., 2006). The hallmark of autophagic activation is the formation of autophagosome puncta containing LC3 II, whereas the biochemical measurement of autophagic activity is expressed as the amount of LC3 II that accumulates in the absence or presence of lysosomal activity.

Autophagy was first implicated in the regulation of IB formation and clearance of aggregate-prone proteins based on the use of chemical activators/inhibitors, including the proautophagic drug rapamycin and knockdown of different autophagic genes (Rubinstein, 2006). The finding that rapamycin and its analog CCI-779 protect against neurodegeneration in animal models of misfolding diseases (Ravikumar et al., 2004; Berger et al., 2006) opens up immense hopes for treating debilitating diseases such as the polyQ disorders. Rapamycin, a macrolytic lactone produced by *Streptomyces hygroscopicus*, has immunosuppressive, antimicrobial, and antitumor properties. It binds intracellularly to FK506 binding protein 12 and targets the protein kinase mammalian target of rapamycin (mTOR). Inhibition of phosphorylation of mTOR by rapamycin activates autophagy, and it has been suggested that rapamycin (or analogs) ameliorates neurodegenerative proteinopathies via activation of autophagy (Rubinstein, 2006). However, mTOR has an impact on various downstream targets not necessarily involved in autophagy, including the control of protein synthesis (Dann and Thomas, 2006; Wullschlegel et al., 2006), and because of these effects, it is currently being evaluated in several phase II clinical trials for cancer (Sabatini, 2006). It is therefore unclear whether rapamycin mediates its protective effects solely via autophagy.

To probe the actions of rapamycin on the formation and clearance of expanded polyQ proteins and IBs, we have taken advantage of clonal cell lines of autophagy-proficient (Atg5^{+/+}) and -deficient (Atg5^{-/-}) mouse embryonic fibroblasts (MEFs) that are easily amenable to biochemical and genetic rescue experiments. Using exon 1 of human Htt containing 97 glutamines and fused to enhanced green fluorescent protein (EGFP) (Ex1HttQ97-EGFP) as an aggregation prone model polypeptide, we show that autophagy-deficient cells accumulate insoluble Ex1HttQ97-EGFP more rapidly and form greater numbers of IBs compared with autophagy-proficient cells. Re-expression of Atg5 in Atg5-deficient cells reversed this phenotype. Most strikingly, rapamycin reduced the amount of insoluble Ex1HttQ97-EGFP and IBs to a similar degree in both Atg5^{+/+} and Atg5^{-/-} cells. The formation of SDS-insoluble polyQ assemblies is a cooperative process that is highly dependent on the accumulation of a critical mass of the protein (Scherzinger et al., 1999; Colby et al., 2006). We suggest that a major effect of rapamycin is the reduction in protein synthesis required for polyQ aggregation and IB formation to occur.

Materials and Methods

Expression Vectors. Mammalian expression vectors encoding exon 1 of the HD gene with 25 or 97 glutamines fused at the C terminus to an EGFP tag were a gift from Erich Schweitzer and Alan Tobin (Brain Research Institute, University of California, Los Angeles, CA). The mouse Atg5 expression vector and adenovirus mRFP-LC3 have been described previously (Mizushima et al., 2001; Bampton et al., 2005).

Cell Culture, Transfection, Inclusion Load Measurement, and Microscopy. SV-40-transformed MEF from Atg5^{+/+} and Atg5^{-/-} mice (Kuma et al., 2004) were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Sigma, St. Louis, MO), 4.5 g/l glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma) in 5% CO₂ at 37°C. Cells were propagated in 75-cm² flasks and seeded on 12-mm poly(L-lysine)-coated glass coverslips in 24-well plates for fluorescence analysis or directly onto six-well plates for immunoblot/filter-trap analysis. Cells were trypsinized, counted, and seeded at a density of 4×10^4 cells/well in 24-well plates and 3×10^5 cells/well in 6-well plates. After an overnight culture, cells reached 60 to 80% confluence and were transiently transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The transfection medium was replaced with fresh medium after 4 h, and cultures were incubated for a further 20 h in the presence or absence of the following inhibitors: 200 nM rapamycin (Rap; Sigma), 50 nM bafilomycin A1 (BafA1; Sigma), and 0.3 to 0.01 µg/ml cycloheximide (CHX; Sigma). Cultures requiring longer time courses were split 24 h after transfection and reseeded at lower densities for harvesting after 48 to 96 h. Cells were fixed in 4% paraformaldehyde, washed in phosphate-buffered saline, and analyzed using epifluorescence microscopy with an Olympus X-170 microscope (Olympus, Tokyo, Japan). Images were collected using an AstraCam camera and Ultra-View software (PerkinElmer Life and Analytical Sciences, Waltham, MA). Inclusion load was calculated as the proportion of EGFP-expressing cells that contained IBs. At least 200 cells were counted per condition.

Protein Synthesis and Cell Counting. Cells were briefly washed free of methionine to avoid long-term methionine deprivation and labeled for 1 h in methionine-free RPMI medium (Sigma) containing 10% fetal bovine serum and 1.85 MBq of [³⁵S]methionine (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and the appropriate additives. Cells were washed three times in methionine-containing Dulbecco's modified Eagle's medium, protein was precipitated in ice-cold 20% trichloroacetic acid, and after three washes with 5% trichloroacetic acid, the precipitate was dissolved in 15% SDS and radioactivity was measured by scintillation counting. Little tRNA was found in these pellets. Cells were counted after trypsinization using a hemocytometer.

Immunoblotting, Immunocytochemistry, Filter Trap Assay, and Resolubilization with Formic Acid. Cells were either collected with a cell scraper or trypsinized and counted using a hemocytometer before being pelleted and washed in phosphate-buffered saline. Material was prepared for immunoblotting and filter-trap detection according to Wanker et al. (1999). In brief, cells were lysed on ice for 30 min in filter-trap lysis buffer [50 mM Tris-HCl, pH 8.8, 100 mM NaCl, 5 mM MgCl₂, 0.5% (w/v) Nonidet P-40, and 1 mM EDTA] in the presence of Complete protease inhibitors (Roche, Indianapolis, IN). Insoluble material was pelleted by centrifugation at 16,000g for 10 min and resuspended in 100 µl of DNaseI buffer (20 mM Tris-HCl, 15 mM MgCl₂, 0.5 mg/ml DNase I; Sigma) for 2 h at 37°C. Protein concentrations of soluble (supernatant fraction) and insoluble fractions (pellet) were determined using the Bicinchoninic acid kit (Sigma) and bovine serum albumin standards. Between 5 and 30 µg of insoluble material was diluted into 200 µl of 2% SDS, boiled for 5 min, and applied to a 96-well dot-blot apparatus (Bio-Rad Laboratories, Hercules, CA) containing a cellulose acetate membrane with 0.2-µm pore-size (Macherey-Nagel, Bethlehem, PA). Resolubilization of pellets with formic acid was performed according to

Hazeki et al. (2000). Pellets were treated in 100 μ l of 100% formic acid for 1 h at 37°C, vacuum-centrifuged, and solubilized in 1 \times SDS-PAGE sample buffer (see below). Soluble material was supplemented with 4 \times SDS-PAGE sample buffer (1 M Tris-HCl, pH 6.8, 400 mM dithiothreitol, 8% SDS, and 40% glycerol), and 30 μ g was used for analysis by SDS-PAGE (8–12.5%). Membranes were blocked in 5% milk for 1 h and probed with the following primary antibodies: mouse monoclonal anti-GFP (8371-1; BD Biosciences, San Jose, CA) at 1:4000, rabbit polyclonal anti-phospho-S6 (2211; Cell Signaling Technology, Danvers, MA) at 1:1000, mouse monoclonal anti-ERK (M12320; Transduction Laboratories, Lexington, KY) at 1:5000, rabbit polyclonal anti-actin (A2066; Sigma) at 1:1000, and mouse anti-vimentin (V6630; Sigma, 1:40). Immunocytochemical analysis was performed as in Bampton et al. (2005). Rabbit polyclonal anti-LC3 antibodies were gifts from Yasuo Uchiyama (Osaka University Graduate School of Medicine, Osaka, Japan) and Eiki Kominami (Juntendo University School of Medicine, Tokyo, Japan). Rabbit polyclonal anti-Atg5 antibody was described previously (Mizushima et al., 2001). Blots were subsequently probed with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:5000 and visualized with enhanced chemiluminescence detection reagents (Amersham). Immunoblots and dot-blot signals were scanned with a flatbed scanner (HP Scanjet 5470c; Hewlett Packard, Palo Alto, CA), and densitometry was performed using ImageJ software (National Institutes of Health, Bethesda, MD). For ratiometric values, the integrated pixel intensity of each signal was calculated and divided by the signal intensity obtained under control conditions. For each sample, dot plots were repeated at two to three dilutions to ensure that the signal was not saturated, giving rise to a single value used for statistical analysis. Values from several experiments were then used to determine the mean (fold) difference in signal intensity. Input was normalized either according to cell number or protein content. For SDS-insoluble material, in some experiments, a parallel analysis of protein loading was conducted by immunoblotting for histone expression (M.A.K., PhD Thesis; data not shown).

Statistical Analysis. The mean value of replicates within an experiment (duplicates to quadruplicates) was taken as a single value when calculating standard deviations from multiple experiments. Multiple comparisons were made using ANOVA followed by Tukey's honestly significant difference post hoc test, pairwise comparisons were conducted using two-tailed Student's *t* test, and one sample *t* test or 95% confidence intervals were used for calculating the significance of ratiometric values. These values and the number of experiments performed for each result are indicated in the text and figure legends.

Results

Genetic Ablation of Atg5 Increased Ex1HttQ97-EGFP Accumulation and Inclusion Body Formation. We first tested whether the complete genetic ablation of autophagy (Atg5) in cells (MEFs) modulated the accumulation of polyQ-expanded huntingtin (Htt), because previous experiments have been performed with RNAi approaches or not under conditions of Htt synthesis (Iwata et al., 2005b; Shibata et al., 2006). Atg5^{+/+} and Atg5^{-/-} MEFs were transfected with cDNA encoding exon 1 of human Htt containing either 25 glutamines (Ex1HttQ25) or 97 glutamines (Ex1HttQ97) fused to EGFP. No discernible IBs in cells were found in MEFs of either type transfected with Ex1HttQ25-EGFP (Fig. 1). However, cytoplasmic or nuclear IBs were readily formed in Ex1HttQ97-EGFP-expressing cells of both types (Fig. 1). Evidence that Atg5^{+/+} MEFs were proficient to undergo autophagy whereas Atg5^{-/-} MEFs were not was obtained by expression of mRFP-LC3. Figure 1 shows that Atg5^{+/+} MEFs contained several mRFP-

LC3 puncta, whereas mRFP-LC3 expression in Atg5^{-/-} MEFs was evenly diffuse, as shown previously (Bampton et al., 2005). We did not observe colocalization of mRFP-LC3 with IBs in wild-type cells at this time point (24 h).

The proportion of EGFP-expressing cells containing IBs increased over time in both cell types (Fig. 2A). Approximately twice as many Ex1HttQ97-EGFP-positive Atg5^{-/-} MEFs contained IBs compared with Atg5^{+/+} MEFs after 1 to 2 days (Fig. 2A). Nuclear inclusions in approximately 10 to 15% of both types of cells were evident from the fact that nuclear DNA was "vacated" from spots in which the IBs had deposited (Fig. 1, arrows) (for quantification, see Fig. 4). There was no difference in the transfection rate between Atg5^{-/-} and Atg5^{+/+} cells (quantified in Fig. 4), transfection efficiency varying between 50 and 60% in both types of MEFs (see Supplementary Fig. S1 for low-power fluorescent images of cells). It is important to note that at 24 h after transfection, we did not detect any differences in toxicity caused by either Ex1HttQ25-EGFP or Ex1HttQ97-EGFP expression in Atg5^{+/+} and Atg5^{-/-} cells, as assessed by inspection of nuclear abnormalities (approximately 5% of EGFP+ve cells

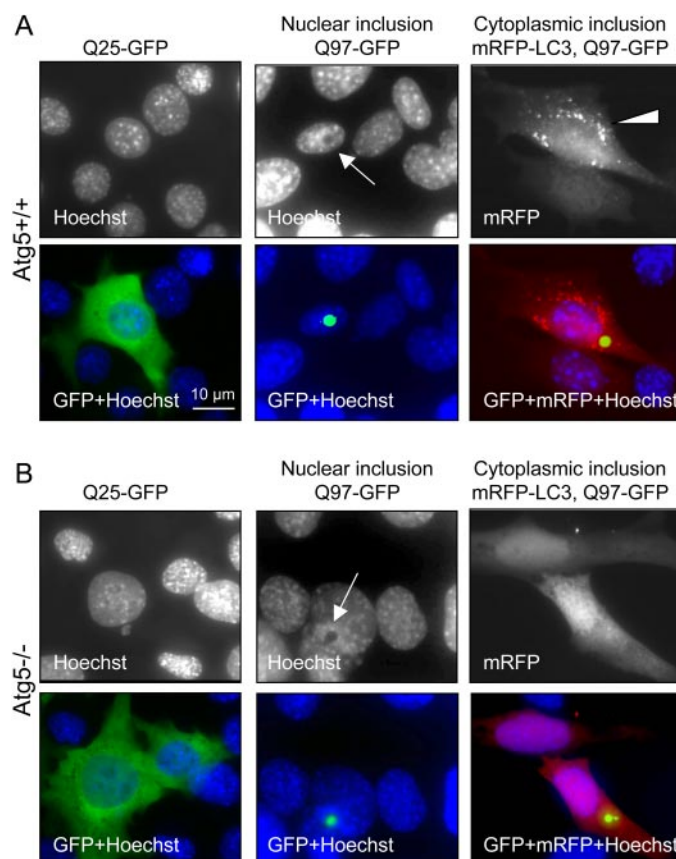


Fig. 1. Atg5^{+/+} and Atg5^{-/-} MEF cells form inclusion bodies containing Ex1HttQ97-EGFP protein, but Ex1HttQ25-EGFP is homogeneously expressed in both cell types. Fluorescent images of Atg5^{+/+} (A) and Atg5^{-/-} (B) MEFs expressing Ex1HttQ25-EGFP or Ex1HttQ97-EGFP 24 h after transfection. The right column shows cells coexpressing mRFP-LC3. Nuclei (blue) were stained with Hoechst 33342. Cells expressing Ex1HttQ25-EGFP do not form IBs and show a homogenous expression (left, green), whereas Ex1HttQ97-EGFP-expressing cells form nuclear (white arrows, middle) and cytoplasmic IBs (right). Note the absence of DNA where a nuclear IB has been deposited (arrow). Only Atg5^{+/+} cells coexpressing Ex1HttQ97-EGFP and mRFP-LC3 (red) show LC3 puncta (right, arrowhead). No localization of mRFP-LC3 to IBs was observed.

showed baseline toxicity as measured by nuclear fragmentation; see Fig. 4 for a quantitative comparison). Therefore the increase in IB formation of Ex1HttQ97-EGFP in *Atg5*^{-/-} cells was due neither to unequal transfection nor to any differential toxicity as a result of IB formation in our experiments.

To test whether the increase in IBs in the *Atg5*^{-/-} cells relative to *Atg5*^{+/+} cells correlated with an increase in the accumulation of SDS-insoluble Ex1HttQ97-EGFP, we used the filter-trap assay to measure the amount of SDS-insoluble Ex1HttQ97 protein formed in each cell type. Pellets remaining after protein extraction in 1% Nonidet P-40 were treated with DNase I, boiled in 2% SDS, and filtered onto a cellulose acetate filter using a dot-blot apparatus (Wanker et al., 1999), whereas respective supernatant proteins were separated by SDS-PAGE. Figure 2B shows that Ex1HttQ97 formed SDS-insoluble material in both types of MEFs, whereas Ex1HttQ25 did not. Quantification showed that there was a 2-fold increase of insoluble Ex1HttQ97-EGFP in *Atg5*^{-/-} cells compared with *Atg5*^{+/+} cells, hence correlating with increased IB formation in autophagy-deficient versus autophagy-proficient cells (Fig. 2D, ■, *n* = 4, *p* < 0.001).

To test for autophagic activity, we probed for LC3 by immunoblotting. Consistent with the lack of mRFP-LC3 puncta in *Atg5*^{-/-} MEFs (Fig. 1B), no LC3 II was detected in *Atg5*^{-/-} extracts from *Atg5*^{-/-} cells immunoblotted for LC3, but extracts from *Atg5*^{+/+} MEFs expressed LC3 II, the latter being the autophagosome-associated form of LC3. A similar amount of LC3 I was expressed in both cell types. Equal input of soluble protein was confirmed with an antibody against ERK1 and ERK2 (tERKs). Further evidence for ongoing autophagy in *Atg5*^{+/+} cells was obtained by treatment with BafA1, which prevents LC3 II degradation in lysosomes and thus causes LC3 II to accumulate in autophagically proficient cells (Kabeya et al., 2000; Bampton et al., 2005). Figure 2C shows that BafA1 significantly increased the amount of LC3 II in *Atg5*^{+/+} cells compared with untreated cells, whereas no changes in LC3 occurred in *Atg5*^{-/-} cells, consistent with the complete absence of autophagy in these cells. Blocking autophagy using BafA1 also significantly increased SDS-insoluble Ex1HttQ97-EGFP in the autophagy-proficient but not -deficient cells (Fig. 2D, quantified in Fig. 2D). The ratio of LC3 II/I varied between experiments (Fig. 2,

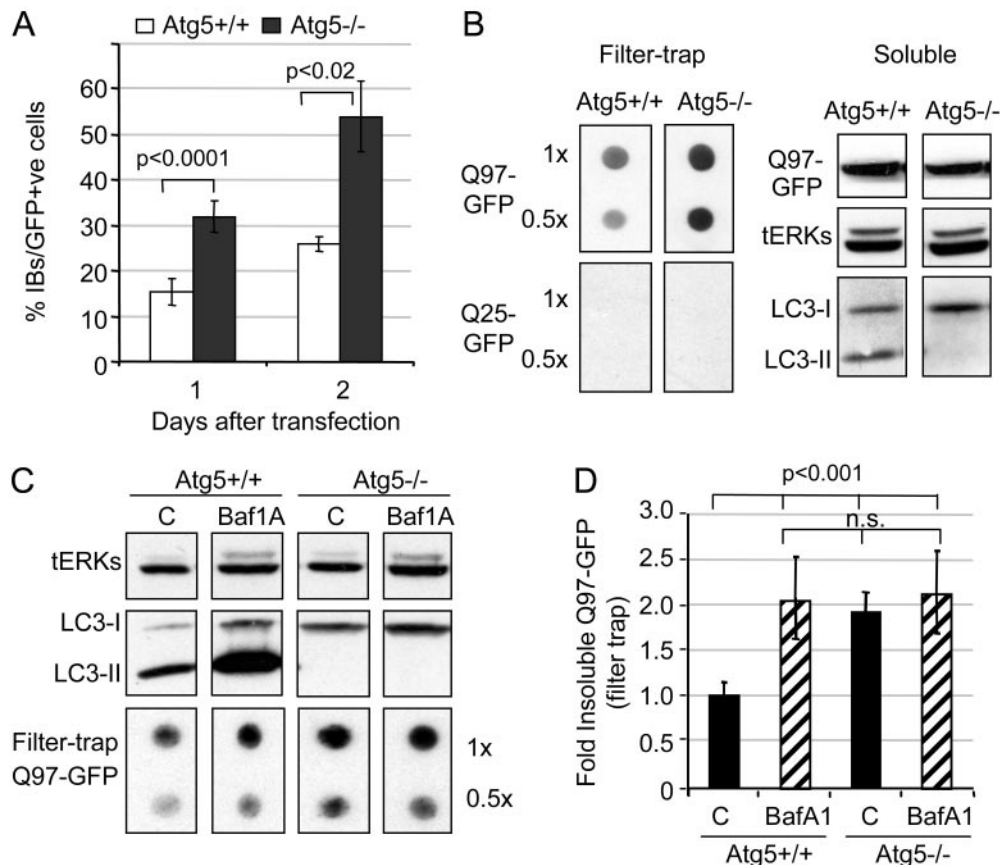


Fig. 2. *Atg5*^{-/-} cells show an increase in the formation of inclusion bodies and SDS-insoluble Ex1HttQ97-EGFP protein. A, the percentage of IBs formed per GFP-positive cells was scored after 1 or 2 days of transfection. *Atg5*^{-/-} cells form IBs at approximately 2-fold the rate of *Atg5*^{+/+} cells up to 2 days after transfection (mean \pm S.D., day 1, *n* = 11; day 2, *n* = 3; *p* = 1.7×10^{-6} by ANOVA, comparisons by *t* test, 1-day *Atg5*^{+/+} versus *Atg5*^{-/-} *p* < 0.0001, 2-day *Atg5*^{+/+} versus *Atg5*^{-/-} *p* < 0.02). B, left, dot blots of SDS-insoluble extracts generated using the filter-trap assay (see Materials and Methods) probed with anti-EGFP; 1 \times and 0.5 \times indicate the relative amount of pellet input. Right, respective soluble extracts probed for GFP (top), tERKs (second row, a loading control), or LC3 I and II (third row). Sample loading for SDS-PAGE and filter-trap assays was normalized to protein content. C, MEFs were treated with 50 nM BafA1 for 20 h after transfection with Ex1HttQ97-EGFP (C, control, no drug treatment), and blots containing equal amounts of soluble protein were probed for LC3 I/II, tERKs (loading control), and SDS-insoluble Ex1HttQ97 (dot blot). The amount of LC3 II increased markedly when *Atg5*^{+/+} cells were incubated in the presence of BafA1, whereas no LC3 II was detected in *Atg5*^{-/-} cells. D, quantification of SDS-insoluble Ex1HttQ97 in *Atg5*^{+/+} and *Atg5*^{-/-} cells (mean \pm S.D., *n* = 4; no significant differences between *Atg5*^{-/-} control, *Atg5*^{-/-} BafA1, and *Atg5*^{+/+} BafA1 conditions by ANOVA, *p* = 0.7, but *p* < 0.001 between the wild-type control value and the other three treatments, Tukey's honestly significant difference). No insoluble material was obtained from either class of MEF cells transfected with Ex1HttQ25-EGFP.

B and C), but BafA1 always increased the amount of LC3 II by at least 2-fold (Fig. 2D). No SDS-insoluble material was detected in extracts of Ex1HttQ25-EGFP transfected cells of either genotype (Fig. 2B). We also did not detect EGFP signals on filters when filtrating the supernatant of Ex1HttQ97-EGFP-expressing cells after spinning at 16,000g (data not shown) but without boiling, suggesting that no SDS-insoluble oligomeric Ex1HttQ97-EGFP species of more than 200 nm (pore size of filter) were generated.

We analyzed whether the IBs are ubiquitinated in both cell types, because this is a hallmark of all polyQ diseases *in vivo*, including HD. We found colocalization of ubiquitin with IBs in ~5% of both Atg5^{+/+} and Atg5^{-/-} cells (Fig. 3 and data not shown). We also found that lysosome-associated membrane protein-1 was associated with IBs in both cell types. Because cytoplasmic IBs are surrounded by intermediate filaments that form an “aggresome” (Waelter et al., 2001), we further probed for the intermediate filament protein vimentin. In both Atg5^{-/-} and Atg5^{+/+} cells, IBs were surrounded by vimentin immunoreactivity. These results show that IBs are qualitatively similar in both cell types.

Together, these data show that Atg5-dependent degradation via autophagy plays an important role in determining the amount of insoluble polyQ-expanded Ex1Htt protein. The decrease in the propensity of cells to form IBs and insoluble Ex1HttQ97-EGFP correlates with their ability to perform autophagy.

Rapamycin Reduced the Amount of Insoluble Ex1HttQ97-EGFP and Inclusion Body Formation in Both Autophagy-Proficient and -Deficient Cells. We next investigated whether rapamycin requires an Atg5-dependent mechanism to modulate the amount of insoluble Ex1HttQ97-EGFP and IB formation. MEFs were treated with 200 nM rapamycin either 12 h before transfection, to instill high

autophagic activity before onset of polyQ expression and IB formation, or were treated with rapamycin simultaneously with transfection. After 18 to 24 h, the percentage of EGFP-positive cells with IBs was determined, whereas insoluble Ex1HttQ97-EGFP was measured using the filter-trap assay as described above. To determine that rapamycin was active, we measured S6 phosphorylation. S6 is a ribosomal protein whose phosphorylation is regulated by S6 kinase in an mTOR-dependent manner (Nobukuni and Thomas, 2004). Figure 4A shows that rapamycin added 12 h before transfection inhibited S6 phosphorylation in both cell types (transfected with Ex1HttQ25-EGFP or Ex1HttQ97-EGFP), indicating that rapamycin prevented mTOR activity independently of Atg5 activity. There was no difference in the amount of Ex1HttQ25-EGFP or Ex1HttQ97-EGFP expressed in either cell type treated with rapamycin compared with untreated cells when the total amount of soluble protein input was equalized between treatments, thus indicating that there is no differential destruction of the transfected proteins *per se* (Fig. 4A).

Pretreatment with rapamycin significantly decreased the proportion of EGFP-positive cells containing Ex1HttQ97-EGFP IBs by 40 to 45% in both Atg5^{+/+} and Atg5^{-/-} MEFs (Fig. 4B). To ensure that rapamycin treatment did not affect the transfection efficiency or toxicity of the Ex1Htt transgenes in either Atg5 cell type, we monitored both. We and others have shown previously that analysis of nuclear morphology as measured by nuclear fragmentation and condensation using DNA stains is a reliable marker of cell toxicity under these conditions and strongly correlates with other markers of cell death (Wytenbach et al., 2001). Figure 4C shows that the toxicity associated with the expression of Ex1HttQ25- or Q97-EGFP was at a baseline level (5%) under our experimental conditions (after 18–24 h after transfection)

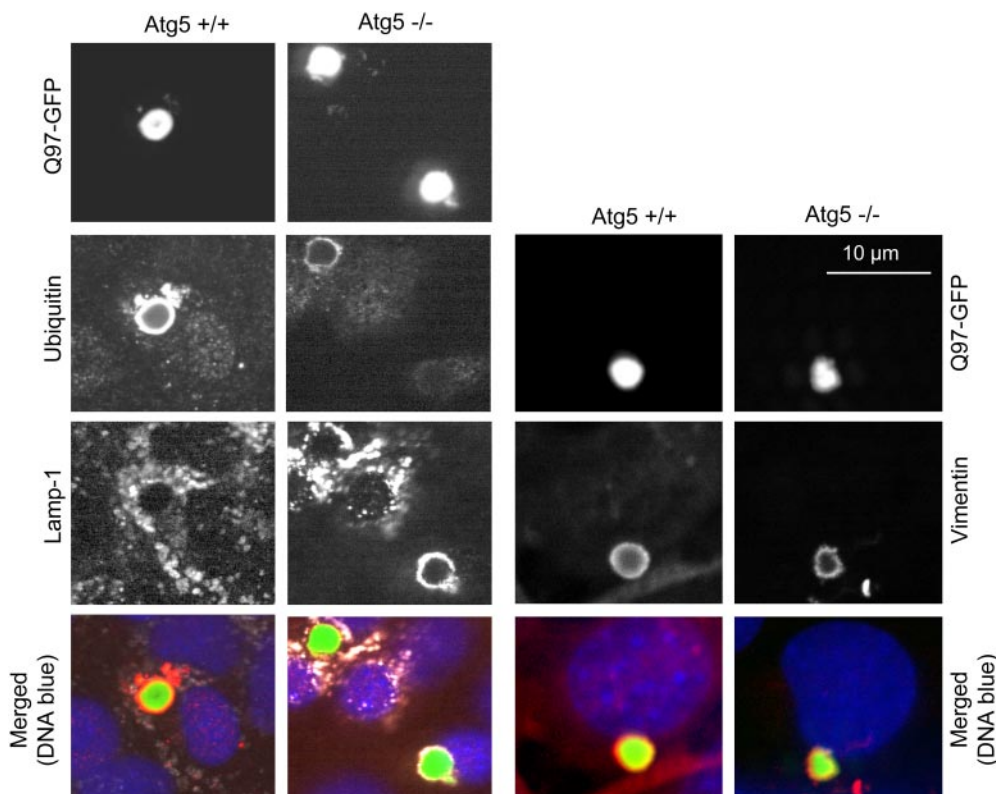


Fig. 3. Colocalization of LAMP1, ubiquitin, and vimentin with IBs. Atg5^{+/+} or Atg5^{-/-} cells were transfected with a construct expressing Ex1HttQ97-EGFP and stained with an anti-ubiquitin antibody (Ubi) and an anti-lysosome-associated membrane protein antibody (Lamp-1). A separate set of cells was stained with anti-vimentin. In both Atg5^{+/+} and Atg5^{-/-} cells, ubiquitin, Lamp-1, and vimentin redistribute toward inclusion bodies formed by Ex1HttQ97-EGFP in ~5% of the cells, showing that IBs seem to be qualitatively similar.

and not different in the two Atg5 cell types (\square). Furthermore, rapamycin treatment did not modulate cell survival compared with control conditions (\blacksquare), demonstrating that the reduction of IBs in both Atg5 cell lines induced by rapamycin (Fig. 4B) was not due to a differential toxicity (mean \pm S.D., $n = 3$, ANOVA, $p = 0.6$). To make sure that the differential increase in IBs between Atg5^{+/+} and Atg5^{-/-} cells (Figs. 2B and 4B) and the decrease in IB formation by rapamycin was not due to unequal transfection rates, we measured the transfection under the various conditions by counting the number of EGFP-positive cells in the total cell population after each experiment in parallel with the analysis of toxicity and IB. As shown in Fig. 4D, we obtained transfection efficiencies of 50 to 60%. It is noteworthy that neither the cell type nor rapamycin affected the rate of transfection (mean \pm S.D., $n = 3$, ANOVA, $p = 0.3$). Because we observed a minor proportion of IBs in the nuclear compartment (Fig. 1), we also quantified the proportion of Atg5^{+/+} and Atg5^{-/-} cells containing IB located in the nucleus versus cytoplasmic localization and whether this distribution is modulated by rapamycin. Figure 4D shows that 10 to 15% of nuclear IBs in both cell types was not changed under rapamycin treatment (mean \pm S.D., $n = 3$, ANOVA, $p = 0.9$).

Having shown that rapamycin treatment reduced IBs in

both cell types, we next investigated whether this reduction was also observed in the amount of insoluble material. IBs are cellular structures (or aggresomes) that may not provide an adequate estimation of the amount of polyQ aggregation. However, we measured a similar reduction in SDS-insoluble Ex1HttQ97-EGFP induced by rapamycin when this was assayed by filter trap, or after solubilizing the SDS-insoluble pellet with formic acid, thereby controlling for equal protein input and loading between the different conditions (Fig. 5A, quantified in B). There was no significant change in the amount of insoluble Ex1HttQ97-EGFP formed in cells that had been treated with rapamycin at the time of transfection compared with control (see supplementary Fig. S2 for raw data). Thus, rapamycin can decrease the amount of insoluble Ex1HttQ97-EGFP and IB load, but this effect occurs in an Atg5-independent manner and with a considerable delay after its addition.

Ravikumar et al. (2004) found that mTOR was inactivated in polyQ-expressing cells, was bound to a polyQ-expanded N-terminal portion of Htt, and sequestered into IBs and thus suggested that autophagy is endemically activated in HD (and maybe other polyQ diseases) as a protective response. In MEFs expressing Ex1HttQ97-EGFP, we failed to find an increase in mTOR immunoreactivity after solubilization of

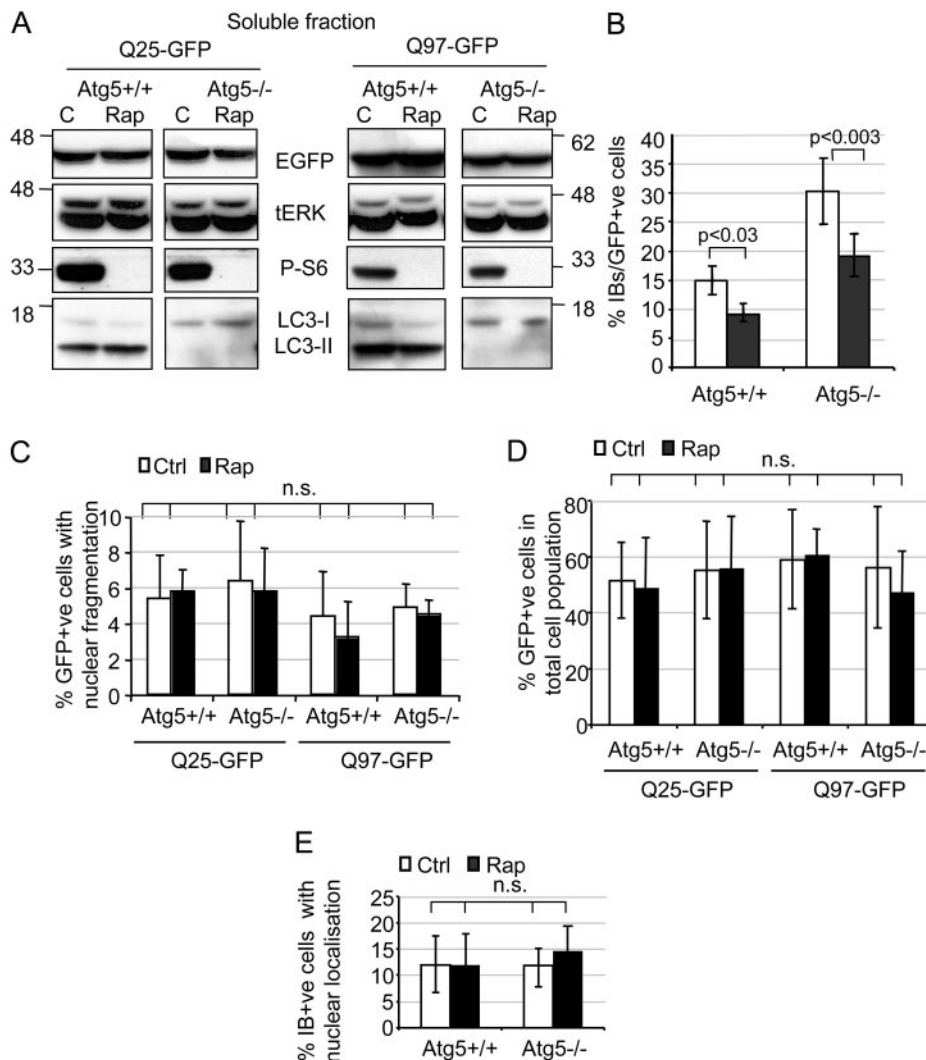


Fig. 4. Pretreatment with rapamycin reduces IB load similarly in both Atg5^{+/+} and Atg5^{-/-} MEFs without affecting toxicity, transfection efficiency, or nuclear distribution of IBs. MEFs were seeded and allowed to attach before culturing in the presence or absence of 200 nM rapamycin overnight (12 h), after which MEFs were transfected with either Ex1HttQ25-EGFP or Ex1HttQ97-EGFP and cultured for a further 20 to 24 h without treatment or in the presence of 200 nM rapamycin before extraction. A, immunoblot of soluble fraction of extracts (see Materials and Methods for an explanation of soluble versus insoluble) normalized to equal protein input. Blots were probed for GFP (top row), tERK (loading control), phosphoserine(235/6)S6, or LC3. No differences were observed in the levels of soluble Ex1HttQ25/97, tERK, or LC3 in response to rapamycin treatment; however, the reduction in S6 phosphorylation shows that mTOR inhibition was achieved. This experiment was repeated four times with similar results. B, the percentage of IBs formed per GFP-positive cell was scored after 1 day (mean \pm S.D., $n = 11$ (control) or $n = 10$ (rapamycin); ANOVA, $p = 5 \times 10^{-6}$, Tukey's post hoc: Atg5^{+/+} untreated versus rapamycin $p = 0.03$, Atg5^{-/-} untreated versus rapamycin $p = 0.003$). C, toxicity was determined by scoring abnormal nuclear morphology using Hoechst 33342 (mean \pm S.D., $n = 3$, ANOVA $p = 0.32$). D, efficiency of transfection was scored by counting the present of EGFP-positive cells (mean \pm S.D., $n = 3$, ANOVA, $p = 0.69$). E, percentage of EGFP-positive cells containing nuclear IB was determined by analyzing fluorescent images (mean \pm S.D., $n = 3$, ANOVA, $p = 0.95$).

IBs using formic acid. mTOR was also not trapped on the stacking gel in conjunction with Ex1HttQ97-EGFP (data not shown), so the degree of sequestration of mTOR may be cell-specific and time-dependent.

Our finding that rapamycin reduced IBs and SDS-insoluble material in autophagy-deficient cells was unexpected. Hence, we next investigated through which mechanism rapamycin reduced IBs and SDS-insoluble Ex1HttQ97-EGFP.

Rapamycin Reduced the Formation of Insoluble Ex1HttQ97-EGFP by Lowering the Amount of Soluble Protein Input. To investigate how rapamycin may be reducing the load of insoluble Ex1HttQ97-EGFP and IBs in Atg5^{-/-} MEFs, the kinetics of cell cycle and protein expression were investigated, because rapamycin is well documented to be a cell cycle suppressant and an inhibitor of protein synthesis (Dann and Thomas, 2006; Sabatini, 2006). To measure this, we counted the number of cells and calculated the amount of decrease due to rapamycin treatment compared with untreated cells set as the control value (presented as percentage of change). Indeed, approximately 25% fewer cells were generated in rapamycin-treated MEFs over the experimental period (36 h) irrespective of Atg5 genetic background or expression of Ex1HttQ25-EGFP or Ex1HttQ97-EGFP (Fig. 6A, left; $0.001 < p < 0.05$ for every condition relative to untreated control; see Supplementary Fig. S3 for raw data). There was no statistical difference in rapamycin-induced decrease in cell number between untransfected cells or cells transfected with Ex1HttQ25-EGFP or Ex1HttQ97-EGFP. The reduction in total cell protein in rapamycin-treated cultures compared with untreated cultures was approximately 37%, irrespective of Atg5 genetic status or Ex1HttQ25-EGFP or Ex1HttQ97-EGFP expression (Fig. 6A, middle; $0.001 < p < 0.05$ for every condition relative to untreated control; see Supplementary Fig. S3 for raw data), and no difference in the amount of this reduction between untransfected or transfected cells with either construct under rapamycin treatment was observed. When the total amount of protein per cell was calculated, the amount of protein was diminished on average by $17 \pm 1.8\%$ in rapamycin-treated cells compared with that in untreated controls, irrespective of genetic background or expression of Ex1HttQ25-EGFP or Ex1HttQ97-EGFP proteins (Fig. 6A, right). Thus, we conclude that rapamycin caused a significant reduction in protein per cell ($p < 0.001$, t test on pooled results, $n = 10$ for each genotype).

To test whether the reduction in total protein per cell due

to rapamycin treatment included protein translated from Ex1Htt-expressing plasmid, we examined the amount of Ex1HttQ25-EGFP produced to avoid the uncertainty associated with the insolubility of Ex1HttQ97-EGFP (Ex1HttQ25-EGFP is soluble under all conditions examined, see above). Figure 6B shows that the average reduction in expression of Ex1HttQ25-EGFP per cell as a result of rapamycin was approximately 14% in both Atg5^{+/+} and Atg5^{-/-} cells, similar to the reduction found in total protein per cell. There was no statistical difference in the reduction of total protein per cell and the reduction of Ex1HttQ25-EGFP per cell as a result of rapamycin treatment. This finding also suggests that Atg5-null cells have no increased protein expression from the plasmids compared with Atg5 wild-type cells because of the lack of the autophagic protein degradation system.

We next measured whether the reduction of 37% of total protein per cell as a result of rapamycin treatment (Fig. 6A) was correlating with a similar reduction in global protein synthesis as measured by direct [³⁵S]methionine incorporation. Thirty hours after the addition of rapamycin, we added [³⁵S]methionine to Atg5^{+/+} cells for 1 h. We observed a significant reduction in [³⁵S]methionine incorporation (Fig. 6C; mean \pm S.D., $n = 3$; t test, $p < 0.001$). We then quantified the reduction as a percentage change under rapamycin treatment compared with untreated cells and found that there was a $39 \pm 3\%$ reduction in [³⁵S]methionine incorporation (Fig. 6D, left bar). This 39% reduction was almost identical with the reduction of $37 \pm 3\%$ found for total soluble protein (Fig. 6D, middle bar) that we calculated in parallel in each of these experiments. Calculating the ratio of change in [³⁵S]methionine incorporation over the reduction of total protein content, this value was no different from 0, indicating that the reduction in the global amount of protein (as shown in Fig. 6, A and D) is probably due to an inhibition of protein synthesis.

Inhibition of Protein Synthesis by Cycloheximide Reduced the Level of Insoluble Ex1HttQ97 and Inclusion Body Formation Similar to Rapamycin. To investigate how a decrease in protein synthesis per cell affects the propensity of cells to form insoluble Ex1HttQ97-EGFP and IBs, a concentration of CHX, an inhibitor of protein synthesis, was sought that matches the decrease in the amount of soluble protein obtained with rapamycin. Cycloheximide was used because it binds to ribosomes reversibly, thus ensuring a response that is proportional to ribosome occupancy over extended periods of treatment. In dose-response experiments

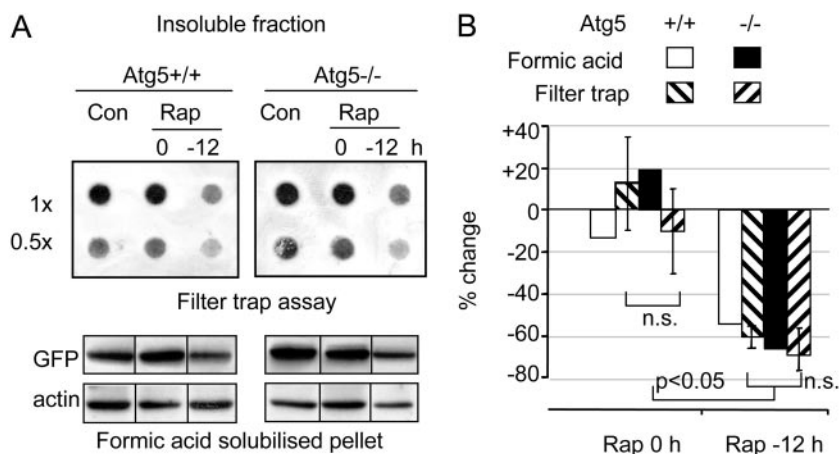


Fig. 5. Pretreatment with rapamycin reduces SDS-insoluble Ex1HttQ97-EGFP in both Atg5^{+/+} and Atg5^{-/-} MEFs. MEFs were seeded and allowed to attach before culturing in the absence or presence of 200 nM rapamycin overnight (-12 h), after which cells were transfected with Ex1HttQ97-EGFP and cultured for a further 20 to 24 h. The sample labeled "Rap 0 h" indicates cells to which rapamycin was added at the time of transfection and maintained during the subsequent 24 h of culture. The SDS-insoluble fraction of the cells were analyzed by filter-trap assay or after resolubilization with formic acid. A, representative example of results from filter-trap assay ($n = 3$) or formic acid resolubilization assay ($n = 1$). B, the percentage of change in SDS-insoluble material induced by rapamycin (mean \pm S.E.M., $n = 6$; $p < 0.05$) for the 12-h pretreatment with rapamycin (-12 h) compared with rapamycin added at 0 time (Rap 0). See Supplementary Fig. S2 for complete data and statistics. Samples used for the filter-trap assay were probed for histone levels, confirming equal loading (data not shown).

using rapamycin, we did not observe a significant difference in the amount of reduction in global protein synthesis between 50 and 200 nM rapamycin (data not shown), and hence, we aimed at a CHX concentration that would match that induced by 200 nM rapamycin.

As expected, the amounts of insoluble Ex1HttQ97-EGFP protein (Fig. 7A) and IB formation (Fig. 7B) were highly dependent on the concentration of CHX used in both cell types. Between 0.03 and 0.1 $\mu\text{g/ml}$ CHX, a similar amount of insoluble Ex1HttQ97-EGFP was detected in the filter trap assay compared with the insoluble material obtained from rapamycin-treated cells (200 nM) irrespective of Atg5 genetic status (Fig. 7A). The amount of IBs formed with rapamycin also closely matched that observed with this concentration range of CHX (Fig. 7B), the amount of insoluble Ex1HttQ97-EGFP and IB formation being equally reduced. Moreover, steady-state protein levels were reduced similarly by the same range of CHX and rapamycin (see Supplementary Fig. S5). Finally, when we compared the amount of [^{35}S]methionine incorporation over 1 h in cells pretreated with rapamycin (200 nM) and cells preincubated with different concentrations of CHX for 24 h, a similar inhibition of [^{35}S]methionine incorporation was found with rapamycin and approximately 0.02 $\mu\text{g/ml}$ CHX (rapamycin, $41.1 \pm 7.2\%$ compared with $49 \pm 4\%$ with 0.02 $\mu\text{g/ml}$ CHX; mean \pm range, two independent experiments). This result suggests that a concentration of CHX that similarly inhibits protein synthesis compared with 200 nM rapamycin also produces equivalent reduction in insoluble Ex1HttQ97-EGFP and IBs obtained by 200 nM rapamycin. Hence, a small reduction in the input of Ex1HttQ97-EGFP has major effects on the kinetics of formation of insoluble Ex1HttQ97-EGFP and IBs. It is interest-

ing that at higher concentrations (0.1–0.3 $\mu\text{g/ml}$), CHX also reduced the amounts of LC3 I/II in Atg $^{+/+}$ and LC3 I in Atg $^{-/-}$ cells to an equivalent extent by the end of the treatment (Fig. 7A), suggesting that LC3 protein is being turned over quite quickly during this time by nonautophagic mechanisms.

Atg5 Overexpression Partially Restored Autophagic Activity in Atg $^{-/-}$ Cells and Reduced Insoluble polyQ-Expanded Ex1Htt. The characteristics of cell lines can diverge rapidly, even when they derive from a common origin. To further investigate the role of autophagy in the control of insoluble Ex1HttQ97-EGFP and IB load, Atg5 was expressed in Atg $^{-/-}$ and Atg $^{+/+}$ cells by transfection. Atg5 expression in Atg $^{-/-}$ cells induced the formation of the Atg5/Atg12 conjugate and restored the ability of the cells to produce LC3 II to approximately 20% of the levels measured in Atg $^{+/+}$ cells (Fig. 8A). It is interesting that we found colocalization of overexpressed Atg5 with IBs (see Supplementary Fig. S4A) and a significant amount of Atg5 (but not Atg5/12 conjugate) accumulated in the pellets of both types of MEF cells expressing Ex1HttQ97-EGFP (Supplementary Fig. S4B shows results for Atg $^{-/-}$ cells). Whether this Atg5 is disabled from performing its function (because no Atg12 accumulated in this fraction) remains to be resolved. Together, these results suggest that some of the overexpressed Atg5 protein in Atg $^{-/-}$ cells is functional, consistent with the recent finding of Hosokawa et al. (2006) using the same cell clone and DNA plasmids.

When the amount of insoluble material was examined using the filter-trap assay (Fig. 8B), overexpression of Atg5 in Atg $^{-/-}$ cells reduced the amount of insoluble material that accumulated each day. Already after 1 day, the amount of

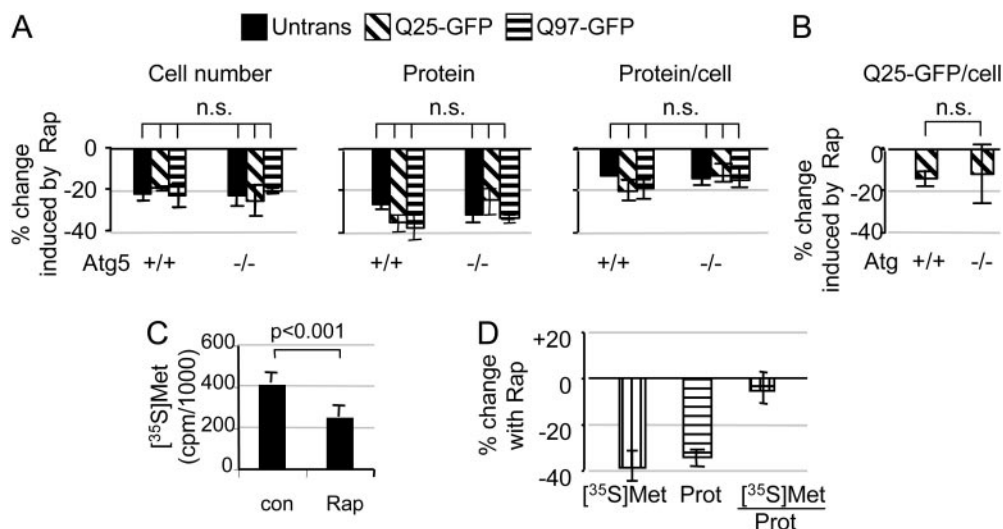


Fig. 6. Rapamycin reduces the amount of protein synthesized per cell. A, Atg $^{+/+}$ and Atg $^{-/-}$ MEF cultures were plated for 12 h in the absence or presence of 200 nM rapamycin, after which two cohorts from each group were transfected with Ex1HttQ25-EGFP or Ex1HttQ97-EGFP and left in the respective media for 24 h. One set of cells was trypsinized and counted to determine cell number (left), whereas total soluble protein was extracted for the other set, and the amount of total soluble protein was determined (middle). Right, the amount of calculated protein per cell. Results are depicted as the percentage of change between rapamycin-treatment and control. Both cell number and the amount of protein was reduced by rapamycin, leading to a 17% reduction in protein per cell, irrespective of the expression of the polyQ proteins. Data show mean \pm S.D.; Q97, $n = 5$, Q25, $n = 2$, untransfected, $n = 3$; cell count, ANOVA $p = 0.83$; Total soluble protein, ANOVA $p = 0.08$; pooled results show that there are no significant differences between the two genotypes, $p \geq 0.2$. See Supplementary Fig. S3 for complete data and statistics. B, cells were pretreated with rapamycin and transfected with Ex1HttQ25-EGFP as in A; the amount of Q25-EGFP per cell was determined by measuring the amount of Q25-EGFP by immunoblotting with an anti-GFP antibody, with the loading normalized to cell number rather than to total protein. Results show mean \pm range from two independent experiments (t test, $p = 0.7$) and demonstrate that rapamycin also reduces protein expression from the plasmid. C, [^{35}S]methionine was added to Atg $^{+/+}$ cells 30 h after the addition of rapamycin, and total methionine incorporation was determined after 1 h as detailed under *Materials and Methods* (mean \pm S.D., $n = 3$, t test $p = 0.001$). D, the percentage of change induced by rapamycin is shown alongside the total amount of protein, which was measured in parallel samples. The right bar shows that the ratio of methionine incorporation to total protein is no different from 0, demonstrating that inhibition of translation is the likely cause of the reduction in the amount of protein per cell.

insoluble Ex1HttQ97-EGFP in $Atg5^{-/-}$ cells expressing $Atg5$ decreased by $27 \pm 8\%$ compared with $2 \pm 1\%$ change in $Atg5^{+/+}$ cells (mean \pm S.E.M., $n = 4$, $p < 0.02$, t test; Fig. 8C). To compare the rate of reduction of insoluble Ex1HttQ97-EGFP under conditions of $Atg5$ overexpression over several days, we split and replated the transfected cells after 2 days of transfection and measured the rate of decrease of Ex1HttQ97-EGFP in both cell types (Fig. 8D). $Atg5$ overexpression accelerated the reduction of Ex1HttQ97-EGFP in $Atg5^{-/-}$ cells but not in $Atg5^{+/+}$ cells. It is important to note that overexpression of $Atg5$ did not reduce the amount of total protein harvested from the $Atg5^{-/-}$ or $Atg5^{+/+}$ cells (unlike rapamycin), suggesting that the decrease in Ex1HttQ97-EGFP caused by $Atg5$ is independent of protein synthesis (data not shown). Moreover, the number of cells harvested at each time point was similar between the four conditions, demonstrating that $Atg5$ overexpression had no effect on the cell cycle. It should be noted that insoluble Ex1HttQ97-EGFP also decreases over time in cells not overexpressing $Atg5$ from days 2 to 4 (Fig. 8, B and D). This is probably due to continuous cell division, which reduces the number of plasmids present in

each cell. A differential loss of cells containing insoluble Ex1HttQ97-EGFP between days 2 and 4 may also contribute to this finding. Together, these data show that re-expression of $Atg5$ in autophagy-deficient cells achieves a reduction in the amount of insoluble Ex1HttQ97-EGFP.

Discussion

In the present study, we showed that autophagy-deficient cells lacking $Atg5$ expression accumulate more misfolded insoluble polyQ protein (Ex1HttQ97-EGFP) and form more IBs than control cells and that this effect can be partially reversed by re-expressing $Atg5$. These data suggest that the lack of autophagy increases polyQ aggregation and/or reduces the clearance of aggregation-prone polyQ proteins. Our results obtained through a genetic approach (complete genetic knockout of $Atg5$) are consistent with those of recent studies (Iwata et al., 2005a,b; Kouroku et al., 2007).

Rapamycin is a well characterized activator of autophagy and has been reported previously to alleviate toxicity of different aggregate-prone proteins (Rubinshtein, 2006). In these reports, the authors suggested that the beneficial effects of rapamycin resulted from its ability to reduce aggregation-prone toxic proteins via autophagy. Hence, one would predict that rapamycin would reduce polyQ aggregation in autophagy-proficient cells but not in autophagy-deficient cells. We tested this idea by treating $Atg5^{+/+}$ and $Atg5^{-/-}$ cells with rapamycin and found that rapamycin reduced soluble and insoluble aggregate-prone Ex1Htt fragments independent of autophagic activity. The evidence for this effect of rapamycin is as follows: 1) rapamycin reduced insoluble Ex1HttQ97 and IB load in $Atg5$ -null cells; 2) inhibition by rapamycin, which rapidly inhibited phosphorylation of S6 kinase within 1 h, was only apparent if the cells were preincubated with rapamycin for approximately 12 h before expression of Ex1HttQ97 for 24 h, a time frame that suggests long-term rather than short-term actions of the drug are required for the effect to occur; 3) rapamycin reduced the amount of soluble protein per cell within this time frame by approximately 17% in both $Atg5^{+/+}$ and $Atg5^{-/-}$ cells, and this amount of inhibition was correlated with the amount of [35 S]methionine incorporated over 1 h at the end of the incubation period, indicating that the reduction in global protein per cell occurred because of a reduction in protein synthesis. The similar extent of rapamycin-induced reduction in the amount of soluble Ex1HttQ25-EGFP per cell indicates that protein synthesis from the plasmid was similarly affected; and 4) the reduction in SDS-insoluble polyQ aggregation and IB formation induced by rapamycin could be mimicked by the use of low concentrations of CHX, which also caused a similar partial reduction in the extent of [35 S]methionine incorporation. Thus, it seems that treatment with rapamycin can have a critical impact on the mass of soluble protein required for polyQ aggregation and IB formation independently of autophagy through a relatively modest reduction in protein input.

It is likely that autophagy too can reduce polyQ aggregation not only through clearance of formed polyQ aggregates but also by reducing the amount of polyQ protein submitted to these processes. These two mechanisms could be acting in an additive way. It is conceivable that some of the effects of

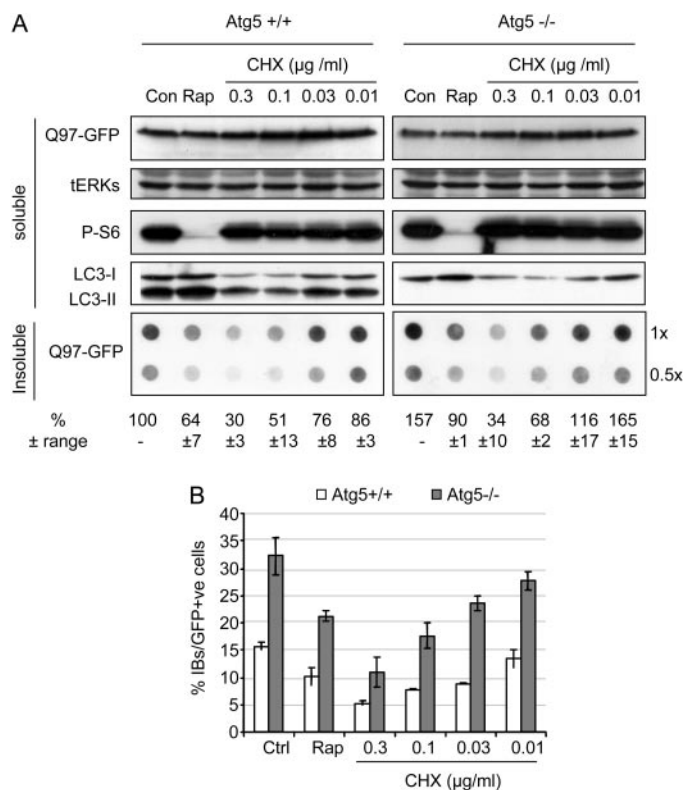


Fig. 7. The reduction in inclusion bodies and insoluble Ex1HttQ97-EGFP induced by rapamycin is mimicked with cycloheximide. $Atg5^{+/+}$ and $Atg5^{-/-}$ MEFs were either pretreated with rapamycin for 12 h or left untreated before transfection with Ex1HttQ97-EGFP. Cells were then cultured with rapamycin or cycloheximide (CHX, 0.01–0.3 μ g/ml) for 20 h before protein extraction. **A**, the soluble fraction was analyzed by immunoblotting for GFP, tERK, or phospho-serine235/6-S6, whereas the insoluble fraction was analyzed by filter-trap assay for presence of Ex1HttQ97-EGFP. The data below the dot blot show mean \pm range of values from two independent experiments. **B**, EGFP+ve cells were scored for IB. **C**, Cycloheximide treatment caused a similar dose-dependent decrease in IBs and SDS-insoluble Ex1HttQ97-EGFP in both $Atg5^{+/+}$ and $Atg5^{-/-}$ cells without affecting soluble levels of Ex1HttQ97-EGFP (**A** and **B**). Note the similarity between the effect of rapamycin (200 nM) and the reduction caused by 0.02 to 0.1 μ g/ml CHX.

rapamycin observed in previous studies may be due to a reduction in protein synthesis. In two studies in which rapamycin was used during the period of Htt synthesis (*Drosophila melanogaster*, transgenic mice) (Berger et al., 2006), it was not reported whether there were changes in expression levels of the transgenes, and hence, it is possible that some of the protective effects were due to the inhibitory action of rapamycin on translation. However, a subtle reduction in the synthesis of an Htt fragment due to rapamycin may be difficult to detect in animal models. Rapamycin treatment has been proposed to inhibit translation of specific mRNAs rather than resulting in global inhibition of translation (Grolleau et al., 2002). However, we found similar amounts of reduction in total protein per cell and in the amount of Ex1HttQ25-EGFP synthesized per cell, suggesting that there is no selectivity in nuclear versus plasmid-based CMV promoter-mediated expression. At present, we cannot distinguish between a possible effect of rapamycin on global translation versus a more specific effect on a subset of mRNAs, although it is clear that the function of some genes required for cell cycle are suppressed.

An interesting question is whether rapamycin mediates all of its effects through mTOR. To date, there are no known alternative targets of rapamycin other than FK506 binding protein 12, which inhibits the protein kinase mTOR (Wullschlegel et al., 2006), suggesting that the inhibitory effects of rapamycin on the synthesis (this study) and the clearance of aggregate prone proteins via autophagy (Rubinsztein, 2006) occur via mTOR. However, not all of the effects of mTOR are mediated by autophagy. The serine/threonine kinase mTOR is a highly conserved integrator of mitogenic and nutrient inputs and controls cell proliferation, cell growth, and endocytosis. These activities are probably mediated through modulation of protein synthesis (Dann and Thomas, 2006; Wullschlegel et al., 2006), consistent with our observation of a reduction in insoluble Ex1HttQ97-EGFP and IBs in the autophagy-deficient Atg5^{-/-} MEFs. The extent of inhibition of protein synthesis of approximately 17% we observed is in keeping with the reported decrease of 15 to 50% in translation rates induced by mTOR inhibition in mammalian cells (whereas in yeast, inhibition is nearer to 100%) (Jefferies et al., 1994; Terada et al., 1994). Hence, it is likely that the

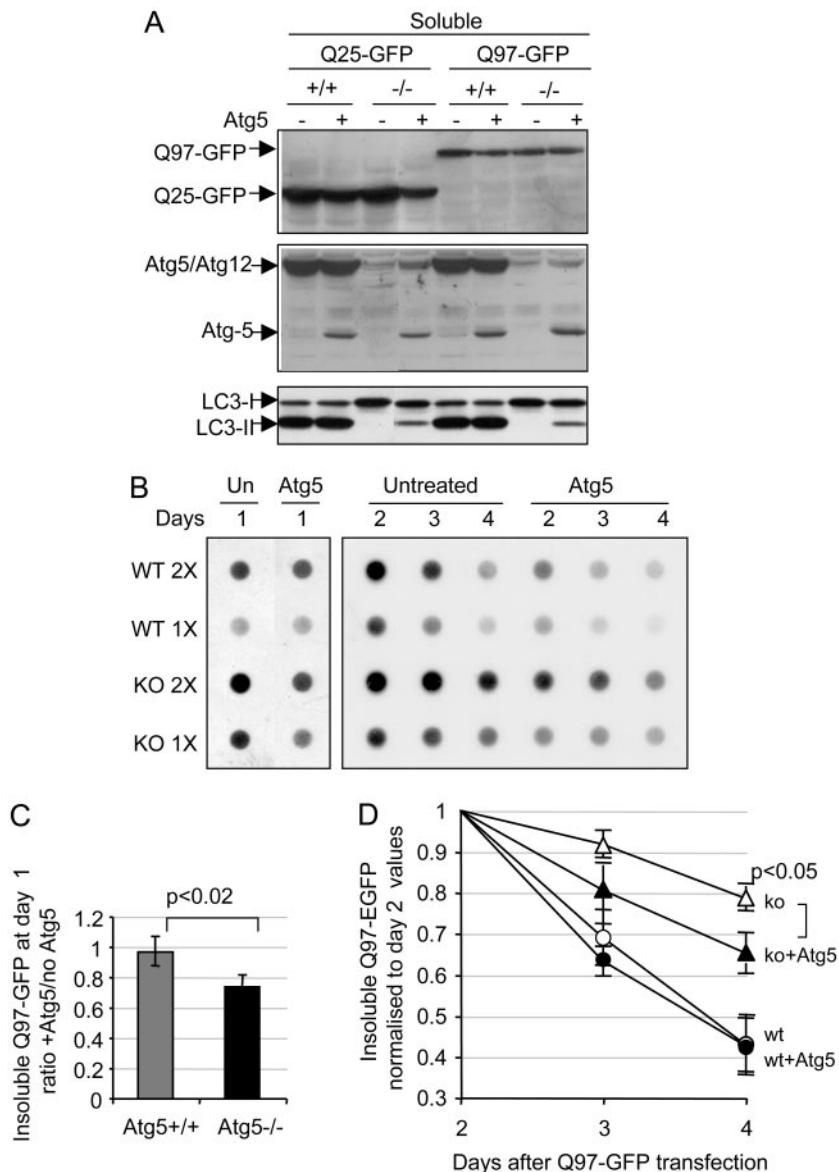


Fig. 8. Atg5 overexpression restores autophagic activity in Atg5^{-/-} cells and reduces SDS-insoluble Ex1HttQ97-EGFP and IB load. Atg5^{+/+} and Atg5^{-/-} MEFs were cotransfected with pcDNA3 or pcDNA3ATG5 and either Ex1HttQ25-EGFP or Ex1HttQ97-EGFP at a ratio of 3:1 and cultured for 24 to 96 h. A, after 48 h of Atg5 expression, the soluble fraction was analyzed by immunoblotting for Ex1HttQ25-EGFP or Ex1HttQ97-EGFP, Atg5, Atg5/12 conjugate, and LC3 I/II. Note that LC3 I processing to LC3 II (and formation of Atg5/12 conjugate) was restored in Atg5^{-/-} MEFs. B, Atg5^{+/+} and Atg5^{-/-} MEFs were cotransfected with pcDNA3 or pcDNA3ATG5 and Ex1HttQ97-EGFP at a ratio of 3:1 and cultured for 24 to 96 h. The blot shows an example of changes in SDS-insoluble material analyzed by the filter-trap assay over 4 days, showing decreased amounts of Ex1HttQ97-EGFP in Atg5^{-/-} cells re-expressing Atg5. Loading of the dot blot was normalized for each condition (30 μ g of protein). C, cells expressing Atg5 for 1 day were analyzed for SDS-insoluble Ex1HttQ97-EGFP. (mean \pm S.E.M., $n = 4$, $p < 0.02$, t test). D, cells were split on day 2 after transfection (so that they could be analyzed for 2 more days without overcrowding), and the amount of insoluble HttEx1Q97-EGFP was quantified at days 2, 3, and 4. Because of varying concentrations of cells between the different experiments, the data were normalized to the values obtained at day 2. Note the increased rate of reduction in insoluble Ex1HttQ97-EGFP in Atg5^{-/-} cells expressing Atg5 compared with untransfected Atg5^{-/-} cells and the lack of effect of Atg5 overexpression in the Atg5^{+/+} cells in C and D.

effect of rapamycin on protein synthesis is cell type-dependent. It remains to be seen to what extent this drug affects protein synthesis in neurons.

The reduction in insoluble Ex1HttQ97-EGFP achieved with rapamycin was mimicked by the use of CHX. Several reports have demonstrated that CHX inhibits autophagy (Kovács and Seglen, 1981) by preventing fusion of autophagosome with endo/lysosomes (Lawrence and Brown, 1993). Consistent with this view, IBs were cleared more quickly in the presence of rapamycin in an inducible cell model after expanded Ex1Htt protein synthesis was turned off, but IBs seemed to accumulate when CHX (10 μ g/ml) was present with rapamycin (Ravikumar et al., 2002). In the above studies, 2 to 10 μ g/ml CHX was used, which inhibits protein synthesis and autophagy more than 95%, whereas in our study, the concentration that mimicked rapamycin's effect was between 0.01 and 0.03 μ g/ml, which elicited only a partial block on protein synthesis and presumably on autophagy. However, our important observation is that the same reduction in insoluble material and IBs were elicited by CHX in Atg5^{-/-} MEFs, in which no autophagy can take place. Hence, the phenomenon we observed here is unlikely to be related to autophagy.

Autophagic clearance of Htt fragments may be promoted independently of mTOR (and S6K/Akt) via a pathway dependent on insulin receptor substrate 2 and stimulated via insulin and IGF-1 (Yamamoto et al., 2006). The notion that there are mTOR-independent pathways of autophagy is suggested by the finding that rapamycin inhibited autophagy suppression by insulin but not by regulatory amino acids in liver cells (Kanazawa et al., 2004), and yeast susceptibility to osmotic stress differed when induction of autophagy was by starvation or rapamycin (Prick et al., 2006). Indeed, it has been shown recently that small-molecule enhancers of mTOR can act independently of rapamycin to enhance the clearance of mutant huntingtin fragments and A53T α -synuclein (Sarkar et al., 2007). These findings suggest that multiple mechanisms may regulate autophagic clearance of misfolded/aggregated proteins. However, irrespective of how the proteins are cleared, a reduction in the critical mass of aggregation-prone proteins such as that described in the present study will always be beneficial in reducing protein aggregation.

Reducing the expression of Htt is, in theory, a promising strategy to alleviate disease: a decrease in the expression of wild-type Htt up to 50% is not detrimental (based on studies in heterozygotes), whereas decreasing the expression of mutant Htt using a short interfering RNA approach has been found to significantly reduce HD pathology in mice (Wang et al., 2005; Machida et al., 2006). This idea is supported by the recent findings of Colby et al. (2006), who proposed that an efficient therapeutic strategy for HD lies in reducing the rate of mutant Ex1Htt aggregation by only modestly reducing Htt expression levels. From our study, we cannot conclude that rapamycin would have a beneficial effect by reducing Htt synthesis in all cases. Future in vivo studies are required to address this point.

It is important to note that in HD in vivo, mutant Htt is constantly produced; hence, the mechanisms by which rapamycin regulates the accumulation and turnover of mutant Htt should be studied under condition of continuous Htt expression as we have done in our cellular model. The stochastic model of Colby et al. (2006) was applied under condi-

tions in which no further protein synthesis was taking place during the misfolding process. If rapamycin can reduce Htt concentrations in HD, it might be beneficial even under conditions in which autophagy is already working at its maximal rate.

Acknowledgments

We thank Dr. Yasuo Uchiyama (Osaka University Graduate School of Medicine, Osaka, Japan) and Dr. Eiki Kominami (Juntendo University School of Medicine, Tokyo, Japan) for the provision of anti-LC3 antibodies, and Drs. Eric Schweitzer and Alan Tobin for the original constructs of Ex1HttQ25-EGFP and Ex1HttQ97-EGFP. Most of this work was conducted in Aviva M. Tolkovsky's laboratory under joint supervision of Andreas Wytenbach and Aviva M. Tolkovsky.

References

- Bampton ETW, Goemans CG, Niranjana D, Mizushima N, and Tolkovsky AM (2005) The dynamics of autophagy visualised in live cells: from autophagosome formation to fusion with endo/lysosomes. *Autophagy* 1:23–36.
- Berger Z, Ravikumar B, Menzies FM, Oroz LG, Underwood BR, Pangalos MN, Schmitt I, Wullner U, Evert BO, O'Kane CJ, et al. (2006) Rapamycin alleviates toxicity of different aggregate-prone proteins. *Hum Mol Genet* 15:433–442.
- Colby DW, Cassidy JP, Lin GC, Ingram VM, and Witterup KD (2006) Stochastic kinetics of intracellular huntingtin aggregate formation. *Nat Chem Biol* 2:319–323.
- Cummings CJ and Zoghbi HY (2000) Trinucleotide repeats: mechanisms and pathophysiology. *Annu Rev Genomics Hum Genet* 1:281–328.
- Dann SG and Thomas G (2006) The amino acid sensitive TOR pathway from yeast to mammals. *FEBS Lett* 580:2821–2829.
- Grolleau A, Bowman J, Pradet-Balade B, Puravs E, Hanash S, Garcia-Sanz JA, and Beretta L (2002) Global and specific translational control by rapamycin in T cells uncovered by microarrays and proteomics. *J Biol Chem* 277:22175–22184.
- Hazeki N, Takamoto T, Goto J, and Kanazawa I (2000) Formic acid dissolves aggregates of an N-terminal huntingtin fragment containing an expanded polyglutamine tract: applying to quantification of protein components of the aggregates. *Biochem Biophys Res Commun* 277:386–393.
- Hosokawa N, Hara Y, and Mizushima N (2006) Generation of cell lines with tetracycline-regulated autophagy and a role for autophagy in controlling cell size. *FEBS Lett* 580:2623–2629.
- Iwata A, Christianson JC, Bucci M, Ellerby LM, Nukina N, Forno LS, and Kopito RR (2005a) Increased susceptibility of cytoplasmic over nuclear polyglutamine aggregates to autophagic degradation. *Proc Natl Acad Sci U S A* 102:13135–13140.
- Iwata A, Riley BE, Johnston JA, and Kopito RR (2005b) HDAC6 and microtubules are required for autophagic degradation of aggregated huntingtin. *J Biol Chem* 280:40282–40292.
- Jana NR and Nukina N (2003) Recent advances in understanding the pathogenesis of polyglutamine diseases: involvement of molecular chaperones and ubiquitin-proteasome pathway. *J Chem Neuroanat* 26:95–101.
- Jefferies HB, Reinhard C, Kozma SC, and Thomas G (1994) Rapamycin selectively represses translation of the "polypyrimidine tract" mRNA family. *Proc Natl Acad Sci U S A* 91:4441–4445.
- Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, and Yoshimori T (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* 19:5720–5728.
- Kanazawa T, Taneike I, Akaishi R, Yoshizawa F, Furuya N, Fujimura S, and Kadowaki M (2004) Amino acids and insulin control autophagic proteolysis through different signaling pathways in relation to mTOR in isolated rat hepatocytes. *J Biol Chem* 279:8452–8459.
- King MA (2007) Neurotoxicity and autophagic turnover of huntingtin exon 1 containing expanded polyglutamine. Ph.D. Thesis, University of Cambridge, Cambridge, UK.
- Kourouk Y, Fujita E, Tanida I, Ueno T, Isoai A, Kumagai H, Ogawa S, Kaufman RJ, Kominami E, and Momoi T (2007) ER stress (PERK/eIF α phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. *Cell Death Differ* 14:230–239.
- Kovács AL and Seglen PO (1981) Inhibition of hepatocytic protein degradation by methylaminopurines and inhibitors of protein synthesis. *Biochim Biophys Acta* 676:213–220.
- Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, Ohsumi Y, Tokuhisa T, and Mizushima N (2004) The role of autophagy during the early neonatal starvation period. *Nature* 432:1032–1036.
- Lawrence BP and Brown WJ (1993) Inhibition of protein synthesis separates autophagic sequestration from the delivery of lysosomal enzymes. *J Cell Sci* 105:473–480.
- Machida Y, Okada T, Kurosawa M, Oyama F, Ozawa K, and Nukina N (2006) rAAV-mediated shRNA ameliorated neuropathology in Huntington disease model mouse. *Biochem Biophys Res Commun* 343:190–197.
- Mizushima N, Yamamoto A, Hatano M, Kobayashi Y, Kabeya Y, Suzuki K, Tokuhisa T, Ohsumi Y, and Yoshimori T (2001) Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. *J Cell Biol* 152:657–668.
- Muchowski PJ and Wacker JL (2005) Modulation of neurodegeneration by molecular chaperones. *Nat Rev Neurosci* 6:11–22.
- Nobukuni T and Thomas G (2004) The mTOR/S6K signalling pathway: the role of the

- TSC1/2 tumour suppressor complex and the proto-oncogene Rheb. *Novartis Found Symp* **262**:148–154.
- Perutz MF and Windle AH (2001) Cause of neural death in neurodegenerative diseases attributable to expansion of glutamine repeats. *Nature* **412**:143–144.
- Prick T, Thumm M, Haussinger D, and Vom Dahl S (2006) Deletion of HOG1 leads to osmosensitivity in starvation-induced, but not rapamycin-dependent Atg8 degradation and proteolysis: further evidence for different regulatory mechanisms in yeast autophagy. *Autophagy* **2**:241–243.
- Ravikumar B, Duden R, and Rubinsztein DC (2002) Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. *Hum Mol Genet* **11**:1107–1117.
- Ravikumar B, Vacher C, Berger Z, Davies JE, Luo S, Oroz LG, Scaravilli F, Easton DF, Duden R, O’Kane CJ, et al. (2004) Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nat Genet* **36**:585–595.
- Ross CA and Poirier MA (2004) Protein aggregation and neurodegenerative disease. *Nat Med* **10**:S10–S17.
- Rubinsztein DC (2006) The roles of intracellular protein-degradation pathways in neurodegeneration. *Nature* **443**:780–786.
- Sabatini DM (2006) mTOR and cancer: insights into a complex relationship. *Nat Rev Cancer* **6**:729–734.
- Sarkar S, Perlstein EO, Imarisio S, Pineau S, Cordenier A, Maglathlin RL, Webster JA, Lewis TA, O’Kane CJ, Schreiber SL, et al. (2007) Small molecules enhance autophagy and reduce toxicity in Huntington’s disease models. *Nat Chem Biol* **3**:331–338.
- Scherzinger E, Sittler A, Schweiger K, Heiser V, Lurz R, Hasenbank R, Bates GP, Lehrach H, and Wanker EE (1999) Self-assembly of polyglutamine-containing huntingtin fragments into amyloid-like fibrils: implications for Huntington’s disease pathology. *Proc Natl Acad Sci U S A* **96**:4604–4609.
- Shibata M, Lu T, Furuya T, Degterev A, Mizushima N, Yoshimori T, MacDonald M, Yankner B, and Yuan J (2006) Regulation of intracellular accumulation of mutant Huntingtin by Beclin 1. *J Biol Chem* **281**:14474–14485.
- Shintani T and Klionsky DJ (2004) Autophagy in health and disease: a double-edged sword. *Science* **306**:990–995.
- Tanida I, Ueno T, and Kominami E (2004) LC3 conjugation system in mammalian autophagy. *Int J Biochem Cell Biol* **36**:2503–2518.
- Terada N, Patel HR, Takase K, Kohno K, Nairn AC, and Gelfand EW (1994) Rapamycin selectively inhibits translation of mRNAs encoding elongation factors and ribosomal proteins. *Proc Natl Acad Sci U S A* **91**:11477–11481.
- Waelter S, Boeddrich A, Lurz R, Scherzinger E, Lueder G, Lehrach H, Wanker EE (2001) Accumulation of mutant huntingtin fragments in aggresome-like inclusion bodies as a results of insufficient protein degradation. *Mol Biol Cell* **12**:1393–1407.
- Wang YL, Liu W, Wada E, Murata M, Wada K, and Kanazawa I (2005) Clinico-pathological rescue of a model mouse of Huntington’s disease by siRNA. *Neurosci Res* **53**:241–249.
- Wanker EE, Scherzinger E, Heiser V, Sittler A, Eickhoff H, and Lehrach H (1999) Membrane filter assay for detection of amyloid-like polyglutamine-containing protein aggregates. *Methods Enzymol* **309**:375–386.
- Wullschlegel S, Loewith R, and Hall MN (2006) TOR signaling in growth and metabolism. *Cell* **124**:471–484.
- Wytenbach A, Swartz J, Kita H, Thykjaer T, Carmichael J, Bradley J, Brown R, Maxwell M, Schapira A, Orntoft TF, et al. (2001) Polyglutamine expansions cause decreased CRE-mediated transcription and early gene expression changes prior to cell death in an inducible cell model of Huntington’s disease. *Hum Mol Genet* **10**:1829–1845.
- Yamamoto A, Cremona ML, and Rothman JE (2006) Autophagy-mediated clearance of huntingtin aggregates triggered by the insulin-signaling pathway. *J Cell Biol* **172**:719–731.

Address correspondence to: Dr. Andreas Wytenbach, Southampton Neuroscience Group, School of Biological Sciences, University of Southampton. Bassett Crescent East, Southampton SO16 7PX, UK. E-mail: aw3@soton.ac.uk
