

Supplemental Information

The Selective Macroautophagic Degradation of Aggregated Proteins Requires the PI3P-Binding Protein Alfy

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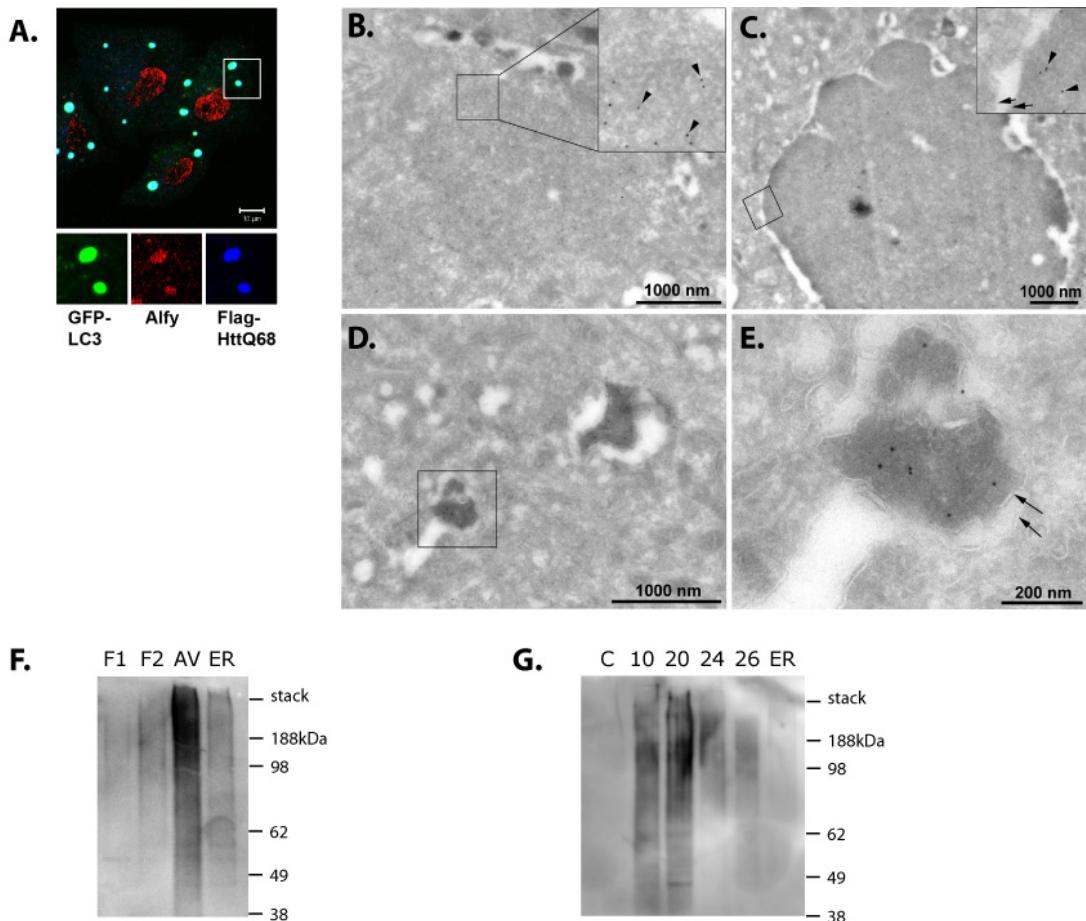


Figure S1. PolyQ inclusions by confocal (A) microscopy, electron microscopy (B-E) and immunoblot. A. GFP-LC3, Alfy and FLAG-exon1htt-Q68 co-localize in inclusions. Cells stably expressing GFP-LC3 were transiently transfected with Flag-exon1httQ68 and probed for FLAG (blue) and endogenous Alfy (red). B-E. Subcellular localization of GFP-HttQ-103 by immuno EM. We observed numerous, cytosolic aggregates of varying diameter in GFP-HttQ-103 expressing cells (B, arrowheads denoting anti-GFP 10nm in all micrographs). Sequestered aggregates could however also be found regularly. In rare cases we could observe very large aggregates (up to 5μm diameter) surrounded by a double membrane structure (double arrow in inset in C) reminiscent of autophagic sequestration membranes. More frequently we found smaller aggregates (below 1μm in diameter) sequestered in double layered structures (D and magnified inset in E). Note again the double membrane structure denoted with double arrows in D. Interestingly the sequestered aggregates had very often a dense appearance and seemed to be contrasted stronger than cytosolic aggregates. Size bars as indicated. F, G. Immunoblotting subcellular fractions for polyubiquitin reveals an enrichment of SDS-soluble and -insoluble polyubiquitinated species in the AV fractions collected using two different methods. F. Nycodenz gradient method. G. Metrizamide gradient method. 10ug of each fraction was loaded on 4-12% SDS PAGE gels, transferred for 3hrs and probed with anti-polyubiquitin. C= cytosolic fraction

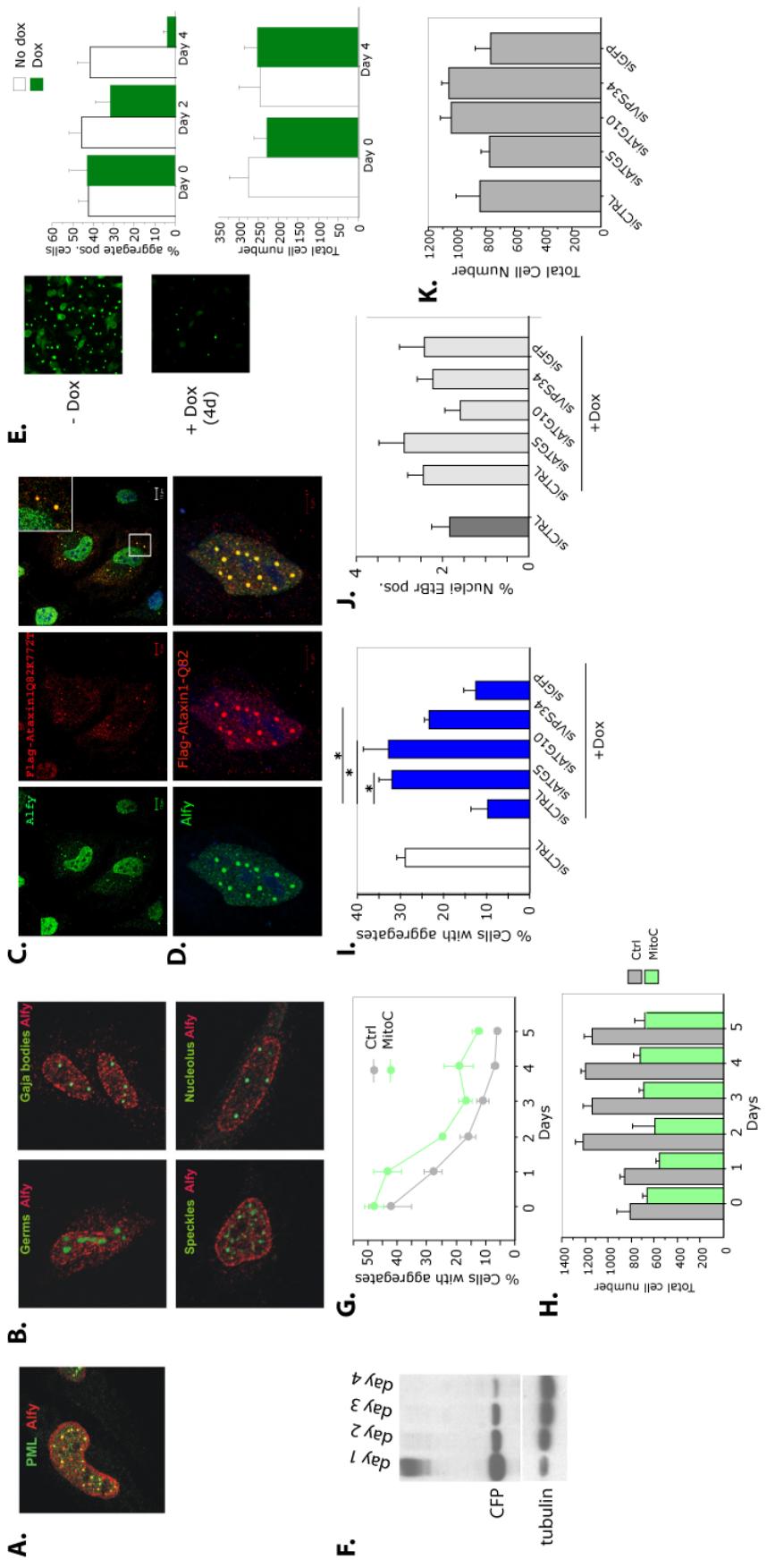


Figure S2. A,B Subcellular localization of Alfyl after LMB inhibition. Alfyl localizes with PML bodies but not with other sub-nuclear structures such as Gaia bodies (anti-collin), Germs (anti-Gemin2), speckles (anti-Sc35) or nucleolus (anti-nucleolin). **C, D** Alfyl co-localizes to intranuclear inclusions. HeLa cells transiently expressing Flag-tagged Ataxin1-Q82-K77T1 (C) or Ataxin1-Q82 (D) (red) and with endogenous Alfyl (green). Nuclei are stained with Draq5 (blue). **E-K HttPolyQ-mCFP stable cell lines is not due to preferential cell death of aggregate-containing cells or to cytoplasmic dilution.** Alfyl aggregates fclear when transgene expression is suppressed. Cells were maintained in the "gene-on" state for 4 passages. 100ng/ml dox was administered into the cell culture media. Cells were fixed at the times indicated, and imaged by confocal microscopy. Clearance of CFP-positive aggregates (green) can be observed after 4d. Representative images shown. The % cells containing aggregates and total cell number were quantified using NIH image. 15 images total were examined (5 images per coverslip, 3 coverslips per time point). **F-H Aggregates are predominantly degraded rather than dissipated upon cytotoxicity.** F. Western blot analysis of whole cell lysates collected everyday for 4 days of dox exposure. To reduce the influence of cell division, the same number of cells was plated at Day1. Each well is lysed daily with equivolumes of buffer. Once all samples are collected, gels were loaded with equivalent volume of lysates. If cell division played a significant role, then the amount of soluble polyQ would decrease as b-tubulin increased. Soluble protein was detected using an antibody against mCFP. 5ul of total cell lysate was loaded per lane (2.5ug to 10ug of total protein). **G,H** Htt103Q-mCFP cell lines still demonstrate aggregate clearance when cell division is chemically slowed. Cells were plated in a 96-well plate then treated with 10ug/ml of Mitomycin C (Mito C) or 0.1% DMSO (Ctrl) for 3h. Cells were washed with complete media then administered dox at the indicated times. **G.** The % of Cells with aggregates significantly diminished across time (ANOVA reveals an effect of 'time' on % Cell with aggregates' ; $p<0.0001$); however impeding cell division slows the rate of clearance, indicating some contribution of cytoplasmic dilution. (ANOVA reveals an effect of 'Mito C' on '% Cell with aggregates' ($p<0.0001$), but no interaction between 'MitoC' and 'time' ($p=0.6210$). **F.** Mito C significantly inhibits cell proliferation (ANOVA reveals an effect of MitoC' on 'Total Cell Number' ($p<0.0001$)). A significant difference of MitoC' on 'Total Cell Number' ($p=0.0001$) was not observed over the time examined (data not shown). **I-K The elimination of inclusions from the Htt103Q-mCFP is independent on macroautophagy.** siRNA-mediated KD of Atg5, Atg10 or Vps34 inhibits clearance of polyQ aggregates, and does not evoke cell death. Experiments were performed as previously described. **I.** 72h post-transfection cells (except for siGFP) were exposed to 100ng/ml dox for 4 days and monitored for clearance. ANOVA reveals a significant effect of 'siRNA' ($p<0.0001$), 'Dox treatment' ($p=0.0004$) on % Cells with aggregates. Fisher PLSD post hoc analysis reveals a significant difference between the following dox treated groups: siCTRL and siATG5 ($p<0.0001$); siCTRL and siVPS34 ($p=0.0047$); and siCTRL and siATG10 ($p=0.0047$). No difference was found between siCTRL and siGFP ($p=0.3570$). Bars represent Mean values of % Cells with aggregates +St.Dev. J. LIVE/DEAD Cell death assay reveals no increased cell death due to siRNA transfection. Bars represent mean values of % nuclei positive for the dye. A positive control of 2% saponin is not shown. **K.** Cell numbers between the different siRNA transfections do not differ suggesting no effect on cell proliferation.

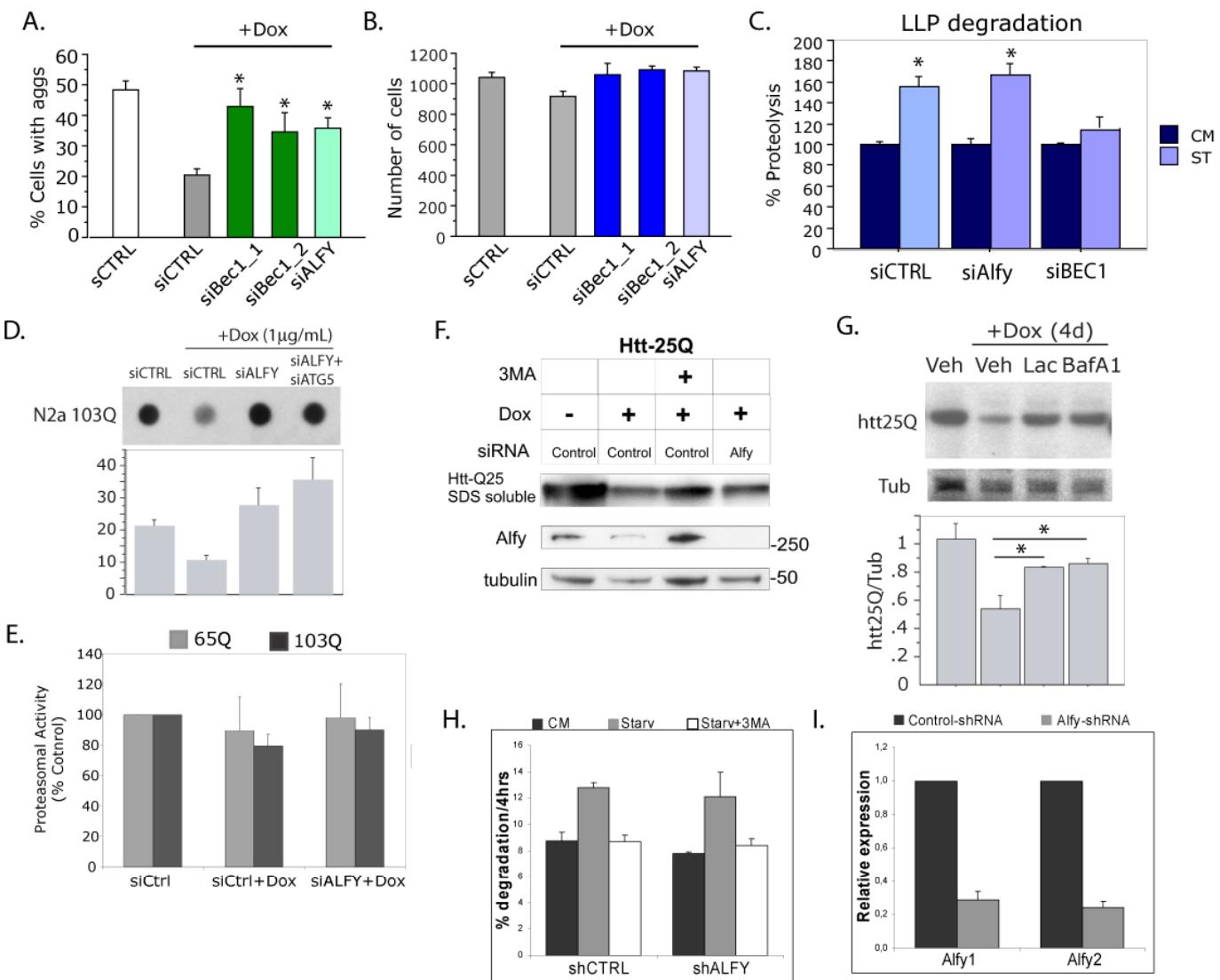


Figure S3: Alfy is required for macroautophagy-mediated clearance but not starvation-mediated LLP degradation. A-C. Mammalian Atg6, Beclin1, is required for the clearance of polyQ inclusions and LLP degradation. A,B. KD of Beclin1 with two different siRNA against (siBec1_1, siBec1_2) in Htt103Q-mCFP cell lines. Bars represent Mean+St.Dev (n=4; >750 cells per well counted). A. 72h treatment with dox leads to a significant clearance of inclusions in cells transfected with a control sequence (siCTRL) ($p<0.0001$). Beclin1 KD significantly impede this clearance. (siBec1_1: $p=0.0008$; *; siBec1_2: $p=0.0068$; *; siALFY: $p=0.0032$, *). ANOVA reveals a significant effect of 'siRNA' on 'PercentCells with Aggregates', on 'Dox Trtmt' and an interaction b/w both variables ($p=0.0080$; $p<0.0001$; $p=0.0437$). Posthoc analysis was performed by Fisher PLSD. B. Cell numbers are not affected by siRNA transfection. ANOVA reveals no significant effect of 'siRNA' on 'Total number of cells' ($p=0.1089$). C. LLP degradation is significantly inhibited after Beclin1 KD. HeLa cells were transfected with siCTRL, siAlfy or siBec1. Bars represent mean+SEM of 3 experiments done in duplicate. ANOVA reveals a significant effect of 'siRNA' and 'Treatment' on 'Percent degradation' ($p=0.012$; $p<0.0001$, respectively), and an interaction between 'siRNA' and 'Treatment' ($p=0.012$). Fisher PLSD posthoc analysis reveals a significant difference between siSCR and siBEC1 ($p=0.024$) and siALFY and siBEC1 ($p=0.005$). No significant difference was seen between siSCR and siAlfy ($p=0.5308$). D. Filter trap analysis of N2a htt103Q-mCFP cells. Cells were transfected with siCTRL, siALFY or siALFY+siATG5. 72h post-transfection a subset of cells were treated with dox for 4d, lysed, pelleted at 500xg and assessed for SDS-insoluble aggregates by filter trap. Bars represent Mean integrated density corrected for background + St.Dev from 2 independent experiments. ANOVA analysis reveals a significant effect of siRNA transfection ($p=0.0226$). A significant inhibition of clearance was observed due to siALFY ($p=0.0206$) and siALFY+siATG5 ($p=0.0056$). No significant difference was seen due to additional inhibition by siATG5 ($p=0.1602$). E. Alfy KD has no significant impact on proteasomal activity in Htt65Q- ($p=0.35$) or 103Q-mCFP ($p=0.2$) were transfected with siCTRL or siAlfy +/- dox and chymotrypsin-like activity was determined as described in Supplemental Methods. Activity is represented as percent control. Bars represent the mean across 3 independent experiments (done in duplicate) + St.Dev. F. Alfy KD has no effect on elimination of soluble Htt25Q-mCFP. Stable cell lines inducibly expressing HttQ25-mCFP were transfected with siCTRL or siAlfy. 48h later, cells were exposed to 100 ng/mL dox for 3 days, then run on SDS-PAGE. G. Htt25Q-mCFP clearance can be influenced by the proteasome inhibitor lactacystin (Lac) and lysosome inhibitor BafA1. Cells were treated with +/- dox for 4d in the presence of Veh, Lac (100nM) or Leu (10ug/mL). Bars represent Mean+StDev from two independent experiments. *: $p<0.05$. H. Cells stably transfected with shRNA against Alfy also exhibit a normal response to starvation when measuring LLP degradation. A. HeLa cells stably transfected with a control-shRNA or Alfy-shRNA are deprived of amino acids for 4h. Bars represent mean of '% degradation' across 3 experiments, duplicates for each condition per experiment. ANOVA detects no significant difference between shCtrl and shAlfy. I. Alfy KD was verified by qRT-PCR using two different primer sets for Alfy. Two different house-keeping primer sets (TBP and SDHA) were used to calculate relative expression levels. Western blotting for Alfy was also performed (data not shown).

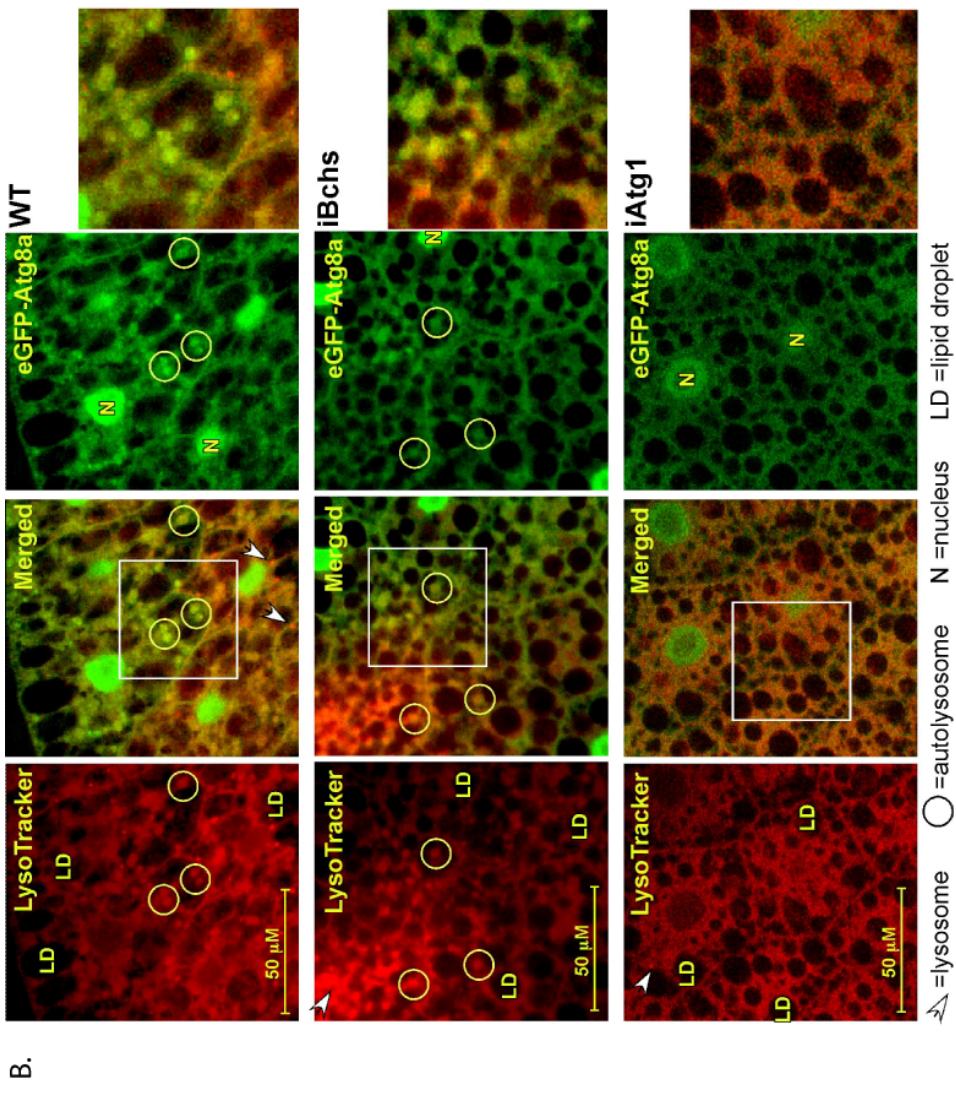


Figure S4. Lysotracker Red (LR) accumulation in the *Drosophila* fat body in response to starvation. A. Loss of *Bchs* (*bchs⁷*) responds similarly to WT control (See in conjunction with Fig.2e). **B.** The fly line containing the Cg-Gal4 driver and UAS-GFP-Atg8a transgene (2nd chromosome, Cg-Gal4, UAS-GFP-Atg8a) has been previously reported and for these experiments was individually crossed with the Canton-S, *bchs*-RNAi (VDRC # 45028) and Atg1-RNAi (VDRC #16133) fly lines. Wandering third instar larvae were collected and fat body tissue fixed (3.7% formaldehyde, PBS) and stained with Lysotracker Red following established protocols (1-3). Tissues were rinsed and mounted in Vectashield and imaged using a Leica confocal fluorescent microscope. Images were processed and composite figures produced using Adobe Photoshop-CS3 and Canvas-X imaging software. In control (WT) and tissue where the *bchs* message has been suppressed (*iBchs*) a robust autophagic response is detected via presence of GFP-Atg8a (green) and Lysotracker (red) positive puncta. In tissue where Atg1 mRNA levels are reduced and autophagy suppressed (*iAtg1*), GFP positive vesicles are absent and few Lysotracker positive structures were detected (arrow). The co-localization of GFP-Atg8a and Lysotracker red is consistent with previous studies (circles) (1-3). It also indicates that Lysotracker staining provides an accurate assessment of autophagic response and vesicle populations in non-transgenic flies and tissues.

1. Lindmo K et al. Exp Cell Res 312:2018-2027, 2006; 2. Rusten TE et al. Dev Cell 7:179-192, 2004; 3. Scott RC et al. Dev Cell 7:167-178, 2004.

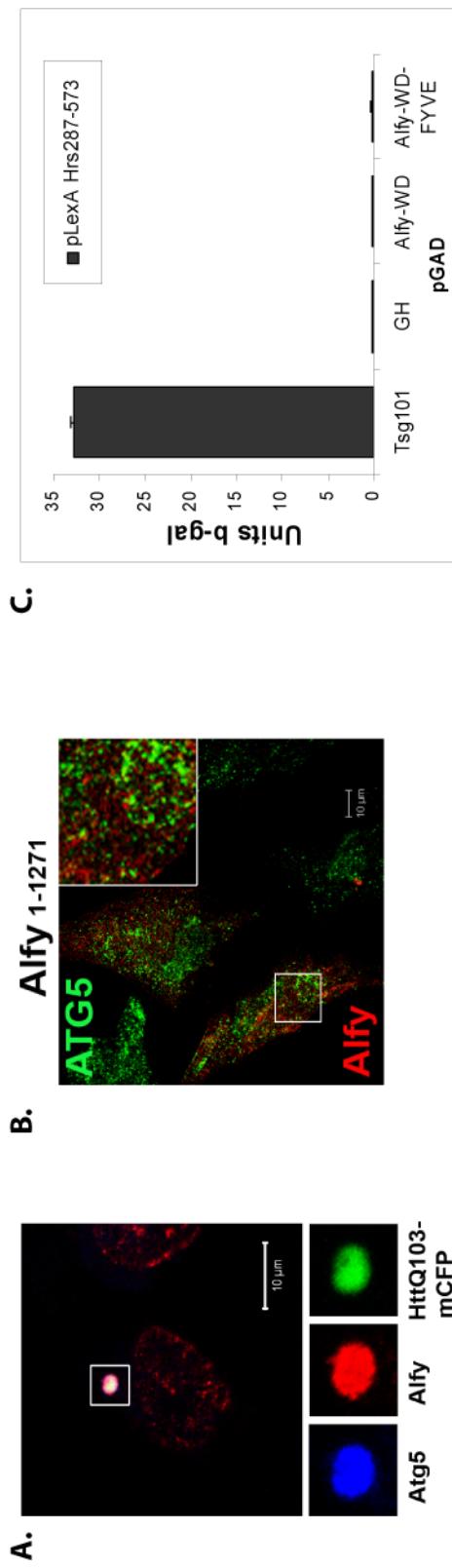
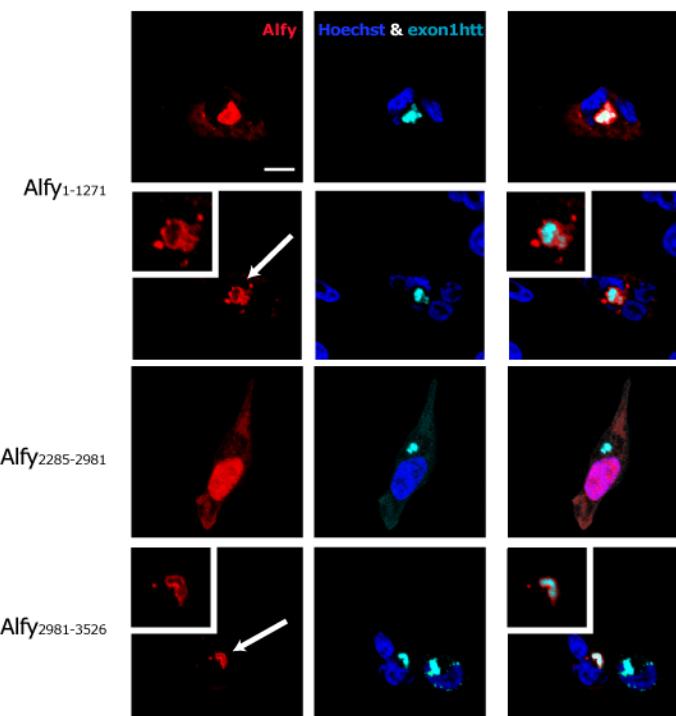
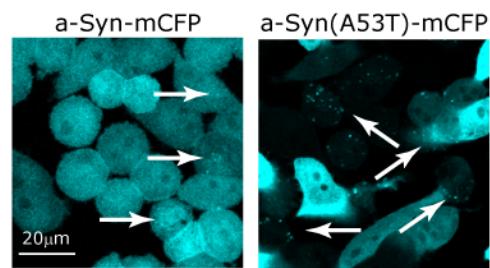


Figure S5. Alfyl localizes to htt inclusions with members of the macroautophagic machinery and directly interacts with Atg5. **A. Endogenous Alfyl and Atg5 co-localize simultaneously with the htt inclusion.** HeLa cells stably expressing HttQ103-mCFP were stained with antibodies recognizing Atg5 (blue) and Alfyl (red), and colocalization was visualized using confocal immunofluorescence microscopy. **B. Overexpression of a tomato-tagged N-terminus fragment of Alfyl fails to co-localize with endogenous Atg5.** In contrast a C-terminus fragment, specifically the WD40 domain, co-localizes with Atg5 in cells (Fig.4a). **C. Yeast two hybrid data demonstrates that the WD40 domain of ALFY directly interacts with ATG5:** Beta-gal assay results in Figure 3d are not due to non-specific interaction. pGAD-ALFY-WD and pGAD-ALFY-WD-FYVE were transformed with pLexA-Hrs287-573, which is known not to interact with Alfyl. No Beta-gal signal was seen. In contrast co-transformation of Tsg101 and pLexA-Hrs287-573 leads to a robust Beta-Gal signal. pGAD control (GH) was co-transformed with pLexA-Hrs287-573 as a negative control.

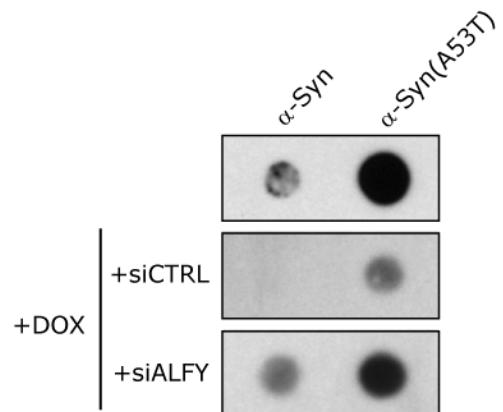
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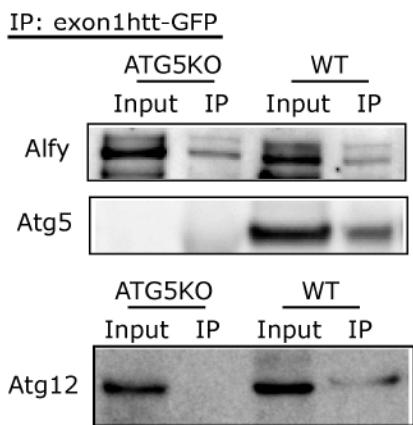
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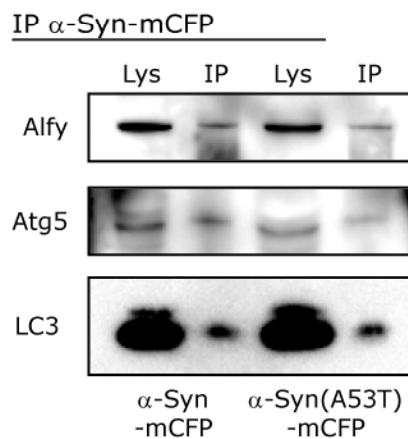


Figure S6. Alfy interacts with both expanded polyQ and alpha-synuclein. **A.** Both an N-terminal and C-terminal region of Alfy co-localizes with the aggregating mutant exon1htt. Stable cell lines (cyan) were transiently transfected with the monomeric tdTomato-tagged Alfy constructs (red) and images were taken by confocal microscopy. Nuclei were stained with Hoechst 33342 (dark blue). Both Alfy N- and C-terminus regions were found to surround the inclusions, while the N-terminus alone co-localized with the inclusion. An internal fragment of Alfy shows no co-localization with exon1htt. **B. Alfy can IP with polyQ proteins in the absence of ATG5.** WT and KO MEFs were transduced with lentivirus carrying the exon1htt72Q tagged to EGFP. The polyQ protein was IPed using the GFP tag. The same immunoblots were probed for Alfy and Atg5. In a separate experiment, Atg12 could also be co-IPed by GFP. **C-D. Alfy is required for the clearance of aggregated a-Syn and a-Syn(A53T).** C. Conditional cell lines expressing mCFP-tagged a-Syn and a-Syn(A53T) accumulate and aggregate (white arrows). D. Filter trap analysis shows detergent resistant aggregates present. Turning off gene expression for 5d with dox reveals the elimination of aggregated protein. Similar to expanded polyQ proteins, depletion of Alfy (siALFY) inhibits this clearance. **E. a-Syn and a-Syn(A53T) can be found in a complex with Alfy, Atg5 and LC3.**

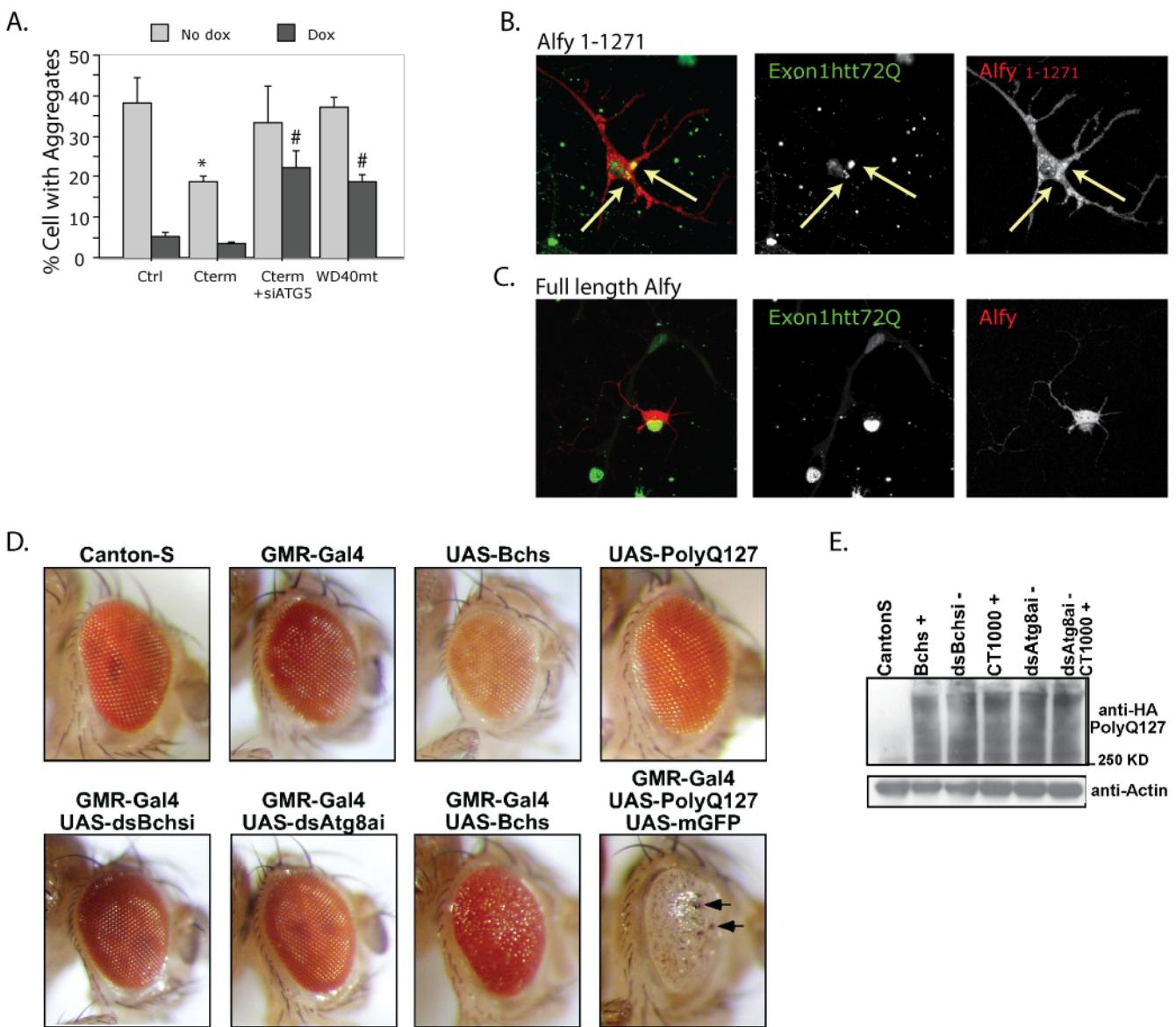


Figure S7. Alfy over-expression enhances clearances of polyQ proteins. A. Atg5 or an intact WD40 domain is required for the enhanced clearance of polyQ aggregates by C-terminal Alfy. Htt103Q-mCFP cells were transiently transfected with tdTomato alone (Ctrl), tdTomato Alfy2981-3526 (Cterm), Cterm+siATG5 or C-termwith 4 W to A mutations in the WD40 domain (WD40mt). Bars represent Mean+St.Err. Each well of transfected cells was split across two wells, of which one was treated with dox. Cells were examined 5ds later and assessed for the Percent (%) of cells with aggregates. In cells not treated with dox, expression of the C-term led to a significant decrease in the %cells with aggregates (*; p=0.0087). This effect was lost in after ATG5 depletion (p=0.4916), or over-expression of WD40mt (p=0.8784). In the presence of dox, siATG5 and WD40mt cells displayed significantly less clearance (#, p=0.0001 and p=0.0002, respectively). ANOVA reveals a significant effect of 'Group' and 'Dox treatment' on 'PercentCells with Aggregates' ($F(3,33)=6.628$; $p=0.0013$ and $F(1,27)=44.157$; $p<0.0001$, respectively) and an interaction between 'Group' and 'Dox treatment'($F(3,33)=2.904$; $p=0.0484$). Posthoc analysis was performed by Fisher PLSD. **B-C. Alfy over-expression in a lentiviral model of HD.** Primary rat cortical neurons were transduced with lentiviruses carrying Exon1 htt with 72Q (green) at DIV2, then transfected with either B. Alfy 1-1271 (red) or C. full length Alfy (red). Alfy 1-1271 can also be found in punctate structures that co-localize to htt inclusions. C. Over-expression of full length Alfy appears to diminish aggregate load similar to Alfy 2888-3526 (Fig. 4); however due to the size of Alfy (400kDa), only a few cells were successfully transfected despite multiple attempts,(n=11). **D. phenotypes for different Drosophila genotypes.** The top left image shows the normal pigmentation and ommatidial organization of a wild type fly eye (Canton-S). The next images show eye phenotypes generated by the pGMR-Gal4, UAS-Bchs and UAS-PolyQ127 transgenic insertions. These constructs are all marked with the hsp-mini-white+ gene. All three lines show normal eye sized and organization, with a range of pigmentation patterns that are consistent for their respective genotypes. Driving expression of double stranded RNA sequences against Bchs and Atg8a also has no effect on the eye. Driving the expression of full-length Bchs produces its own independent rough eye phenotype, while over-expression of the UAS-bchs-C1000 transgene does not produce any obvious external defects (data not shown). The bottom right image of the pGMR-Gal4;UAS-PolyQ127 line crossed to the UAS-mGFP stock shows the PolyQ127 peptide still has a pronounced effect on the eye development of F1 offspring when co-expressed with a second independent protein (arrows show necrosis). **E. Western blot analysis for PolyQ127 levels across Drosophila genotypes.** SDS-PAGE reveals high molecular weight species of PolyQ127 when immuno blotted for HA-tagged PolyQ127. Wildtype Canton-S flies were used as control.

Supplemental Experimental Procedures

Antibodies

Primary antibodies were from the following sources: Rb anti-Alfy (as previously described (Simonsen et al., 2004)); Ms anti-GFP (Clontech Laboratories, Roche), Rb anti-GFP (Abcam, Novus); Ms anti-FLAG (Sigma); Ms anti-tubulin (Sigma); Ms anti-myc (9E10 hybridoma, ATCC); Gt anti-Atg5 (Santa Cruz Biotechnology), Rb anti-APG5L (Abgent, Abcam); Rb anti-ATG7 (Abcam), Ms (B&D Transduction Labs), GP or Rb-SQSTM1/p62 (Abgent); Ms anti-NBR1 (Abnova); Ms anti-htt (Chemicon MAB5492, MAB5490, MABEM48); Rb anti-Dnmt (Stressgen); Rb anti-Atg16L (Proscience); Rb anti-LC3B (Abcam (WB), gift from T. Yoshimori (WB), gift from R. Kopito (IF)). HRP-labeled secondary antibodies were from Pierce. Cy2-, Cy3-, and Cy5-labeled secondary antibodies were from Jackson ImmunoResearch Laboratories.

Cell Culture

HeLa cell cultures were maintained as recommended by American Tissue Culture Collection (ATCC) (Manassas, VA). HeLa cells stably expressing a tet-off inducible exon1 of Htt carrying a polyQ expansion of 25,65 or 103 residues, fused to monomeric enhanced CFP at the COOH termini (Htt 25Q-, 65Q- and 103Q-mCFP) were cultured as described and 100 ng/ml doxycyclin (dox) was used to shut off production of new protein. HEK cells stable expressing GFP-LC3 was generously provided by Sharon Tooze. Human HD fibroblasts were obtained from Coriell and maintained as instructed.

Immuno Electron Microscopy

Cells for immuno EM were fixed in 4% formaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, scraped in 1% gelatin, pelleted and further embedded in 10% gelatin. After infusion with 2.3 M sucrose samples were mounted on silver pins and frozen in liquid nitrogen. Sections were cut at -110°C on a Leica Ultracut and picked up with a 1:1 mixture of 2.3 M sucrose and 2.0 M methyl cellulose. Sections were labeled with a rabbit antibody against GFP (a gift from T. Johansen, Tromsø), followed by 10-nm protein A gold (CMC, Utrecht, The Netherlands) and observed in a JEOL-1230 electron microscope (JEOL, Tokyo, Japan) at 60–80 kV. Images were recorded with a Morada digital camera using iTEM (Olympus SIS GmbH, Münster, Germany) software. Samples for plastic embedding were fixed in 2% glutaraldehyde, postfixed in 2% OsO₄, stained with Uranyl acetate and embedded in Epon after dehydration in alcohol series.

Cell Fractionation and Cryo-Electron Microscopy

Nycodenz and Metrizamide cell fractionation methods were performed as previously described (Jeong et al., 2009). Briefly, Htt103QmCFP cell lines were mechanically disrupted and were fractionated using a discontinuous Nycodenz gradient (Stromhaug et al., 1998) or 50% Metrizamide gradient (Marzella et al., 1982). Isolated fractions were then examined by immunoblotting or cryo-EM. For cryo-EM, organelles were imaged in vitreous ice over open holes on a perforated carbon support to preserve all details in the image. Samples were imaged on a Tecnai 20 FEG electron microscope operating at 200 kV, with magnifications from 25,000 to 50,000 and a typical defocus of -3.5 μm.

Filter-Trap Assay

Cells were lysed in a buffer containing 0.5% IGEPAL and insoluble material was collected by centrifugation. The pelleted material was treated with DNase and then solubilized in 2% SDS. The SDS fraction was applied to a dot-blot apparatus containing a cellulose acetate membrane with pore size 0.2μm (Whatman plc, Maidstone, UK) which traps the SDS-insoluble aggregates. To analyze the effect of siRNA treatment on soluble Htt (IGEPAL-soluble fraction) this fraction was analyzed by SDS page and western blotting. The total protein load was normalized to the volume of the soluble fraction. The protein concentration was measured by the DC protein assay reagent (Bio-Rad, Hercules, CA). An anti-GFP antibody was used to detect htt25Q/65Q/103QmCFP. The intensities of the different dots obtained were quantified using the software provided by the ChemiGenius imaging system (Syngene, Cambridge, UK).

Plasmids Construction

The following pDEST-Alfy constructs were made by subcloning the indicated Alfy amino acid fragments into pENTR vectors, followed by an LR reaction using pDEST-Tomato or pDEST-Cherry according to the manufacturer's protocol for the Gateway™ system (Invitrogen, Carlsbad, CA); pDEST-Cherry-Alfy₃₄₃₄₋₃₅₂₆, pDEST-Tomato-Alfy₂₂₈₅₋₃₅₂₆, pDEST-Tomato-Alfy₂₂₈₅₋₂₉₈₂, pDEST-Tomato-Alfy₂₉₈₁₋₃₅₂₆ and pDEST-Tomato-Alfy₁₋₁₂₇₁. pDEST-HttQ68-Flag have been described (Bjorkoy et al., 2005). pcDNA-myc-Alfy₁₋₁₂₇₁ and pcDNA-myc-Alfy₂₅₄₃₋₃₅₂₇ were generated by subcloning of the indicated Alfy fragments, generated by PCR, into pcDNA-myc (Invitrogen, Carlsbad, CA). *pGAD-GH-Alfy*₃₄₃₄₋₃₅₂₆, *pGAD-GH-Alfy*₃₀₆₈₋₃₅₂₆ and *pGAD-GH-Alfy*₂₇₀₀₋₃₅₂₆ were generated by subcloning of the indicated PCR-amplified Alfy fragments in frame with the Gal4 activation domain of pGAD-GH (Clontech Laboratories, Mountain View, CA). hAtg5 was amplified by PCR from the Marathon Ready human brain cDNA (Clontech Laboratories, Mountain View, CA) and subcloned in frame with pcDNA 3-myc (Raiborg et al., JCS 2001) or with the DNA-binding domain of pLexa/pBTM116 (Vojtek et al, 1993).

Human Atg5, LC3-B or Syntaxin-7 were amplified by PCR from the Marathon Ready human brain cDNA (Clontech Laboratories, Mountain View, CA) and subcloned into pGEX-5X (Amersham Pharmacia,Buckinghamshire,UK). Amino acids 2887-3526 of human Alfy was subcloned into pMAL-c2 (New England Biolabs, Ipswich, MA).

Transfection of Small-Interfering RNA (siRNA) Oligonucleotides and Plasmids

HeLa and HttPolyQ-mCFP cells were transfected with ON-TARGETplus SMARTpool small interfering RNAs (siRNA) (Dharmacon, Lafayette, CO) against human Alfy (WDFY3), Atg7 and Beclin1. Results were confirmed by the use of single siRNA against Alfy (Simonsen et al., 2004a) and one single siRNA from the SMARTpool, having the sequence 5'-GGAAAUGCUCCGCCAAUAUU-3' of the sense strand and 5'-PUAUUCGGCGGAGCAUUUCCUU-3' of the antisense strand. Transfection of HeLa cells with siRNA oligonucleotides was performed as described previously (Bache et al., 2003). Briefly, the cells were transfected with 50-100 nM siRNA using Oligofectamine (Invitrogen, Carlsbad, CA) for 3 d, the cells were then re-plated and left for another 2 d before experiments were carried out. Specific protein knockdown was demonstrated by running equal amounts of cell lysate on SDS-PAGE, followed by western blotting using antibodies against Alfy. Knock-down was also confirmed by RT-PCR. HeLa cells were transfected with the indicated plasmids using the Fugene transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's protocol, and were cultured for 48 h to allow protein expression. Cells were then fixed on coverslips for confocal analyses or lysed for protein detection assays.

Immunoprecipitation and Western Blot Analysis

Immunoprecipitation of Atg5 with C-terminus Alfy (Fig 2b): Cells were harvested and lysed in NP40 buffer (125 mM KAc, 25 mM Hepes at pH 7.2, 5 mM EDTA, 2.5 mM MgAc, 0.5 % NP40 and 1mM DTT) containing protease inhibitor cocktail (Roche). The total cell lysates were used for immunoprecipitation with anti-myc antibody at 4°C for 10-12 h. After addition of protein A/G agarose beads (Amersham Biosciences), the incubation was continued for 1 h. Immunoprecipitates were washed three times with lysis buffer and eluted with SDS loading buffer by boiling for 5 min. Immunocomplexes were subjected to SDS-PAGE and western blot analysis with the indicated antibodies.

Immunoprecipitation of Alfy-htt complex. Cells were lysed with ice cold lysis buffer + protease inhibitor cocktail. Collected lysates were spun at 500xg to pellet unlysed cells and nuclei, then immunoprecipitated as follows: HttPolyQ-mCFP cell lines and exon1htt72Q were immunoprecipitated using anti-GFP Magnabeads (Miltenyi Biotec).

To analyze the cellular levels of different proteins, cells were first extracted in ice-cold lysis buffer (50 mM NaCl, 10 mM Tris, 5 mM EDTA, 0.1% SDS, 1% Triton X-100 + protease and phosphatase

inhibitor cocktails), centrifuged (14000 rpm) for 10 minutes and the supernatants collected. Protein concentrations in the soluble fractions were determined and approximately 20 µg of protein per sample was loaded and resolved on 4-20% gradient gels (Pierce, Rockford, IL) followed by electro blotting to Immobilon-P membranes (Millipore, Billerica, MA). The blots were probed with specific antibodies, which were detected using standard ECL reagents. The intensities of the different bands obtained were quantified using the software provided by the ChemiGenius imaging system (Syngene, Cambridge, UK) and relative amounts quantified using tubulin as a loading control.

Confocal Immunofluorescence Microscopy

HeLa cells grown on coverslips, transfected or not with the indicated siRNA or plasmid, were fixed in 3% paraformaldehyde, permeabilized with 0.05% saponin and stained for fluorescence microscopy as described previously (Simonsen et al., 1998). Coverslips were examined using a Zeiss LSM 510 META microscope equipped with a Neo-Fluar 100x/1.45 oil immersion objective. Image processing and analysis were done with Zeiss LSM 510 software version 3.2, and Adobe Photoshop version 7.0 (Adobe Systems, Mountain View, CA).

For siRNA transfected stable cell lines, images were collected on the InCell Analyzer 3000 and analysed with the Raven software as previously described (Yamamoto et al., 2006). Transiently transfected cells and primary neuron were examined using a Leica confocal microscope. Analyses was performed on accompanying software and NIH Image.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from 5×10^5 HeLa cells using the AurumTM Total RNA mini kit (Bio-Rad, CA, USA), according to the manufacturer's instructions. Purity and quantity were measured by optical density. 1 µg total RNA was used for cDNA synthesis using the iScriptTM cDNA Synthesis kit (Bio-Rad). Real-time PCR was performed in parallel 20 µl reactions containing 10 µl 2x QuantiTectTM SYBR[®] Green PCR master mix (Qiagen, CA, USA), 2 µl 10x QuantiTect Primer Assay (Qiagen) and 20 ng cDNA (2 ng were used for actin) in 96-well optical plates. The cycling conditions for the LightCycler480 (Roche) were 95°C for 15 min, 40 cycles of 94°C 15 sec, 55°C 20 sec and 72°C 20 sec. The following pre-validated QuantiTect Primer Assays (Qiagen) were used; Hs_WDFY3_1_SG, Hs_ACTB_1_SG, Hs_TBP_1_SG. Real-time efficiencies were calculated from the slopes of the standard dilution curves. mRNA levels were determined by the method of direct comparison of C_T values ($C_T > 35$ rejected) and relative quantities calculated by the $\Delta\Delta C_T$ equation or transformed into linear form by $2^{-\Delta\Delta C_T}$. Transcripts were normalized to the quantity of Actin and TBP for each condition.

Purification of GST and MBP Fusion Proteins

GST, GST-Atg5, GST-LC3 and GST-Syntaxin-7 and MBP-Alfy_{CT639} were produced in *E.coli* BL-21. The bacteria were lysed with B-per extraction reagent (Pierce) containing protease inhibitors and 1 mM DTT for 30 min at RT. Lysates containing GST-fusion proteins were incubated with pre-washed Glutathione SepharoseTM (GE Healthcare Bio-Sciences AB) for 30 min at RT. The beads were added to a column and washed with buffer A (20mM Tris, 60mM NaCl, pH=7,6, 1 mM DTT). Bound GST-fusion proteins were eluted with buffer A containing 10mM reduced glutathione and dialysed in 20mM Hepes, 140mM NaCl, pH=7,2. Lysate containing MBP-Alfy_{CT639} was incubated with pre-washed Amylose Resin (New England BioLabs) on a column. The resin was washed with buffer B (20 mM Tris pH. 7,5, 200mM NaCl, 1mM EDTA, 1mM DTT with protease inhibitors) and the protein eluted with buffer B containing 10mM maltose. Eluted protein was dialyzed in 25 mM Tris pH 7,5, 150 mM NaCl.

GST Pulldown Assay

5 µg of GST, GST-Atg5, GST-LC3 or GST-Syntaxin-7 were immobilized on pre-washed Glutathione SepharoseTM (GE Healthcare Bio-Sciences AB). The beads were washed with buffer K (125 mM KAc, 25mM Hepes, 2.5 mM MgAc, 5 mM EGTA, 0.5% NP-40, 1 mM DTT) and incubated with 1 µg of MBP-Alfy_{CT639} for 2.5 h at 4°C. The beads were washed 3 times with buffer K and once with buffer K lacking NP-40 and then boiled in 2x sample buffer. Eluted proteins were analyzed by SDS-PAGE,

transferred to a PVDF membrane and bound MBP-Alfy_{CT639} was detected using western blotting with an anti-Alfy antibody. 15 ng MBP-Alfy_{CT639} was loaded as a control.

Yeast Two-Hybrid Analysis

The yeast reporter strain L40 (Vojtek et al., 1993) was cotransformed with pLexa-hAtg5 and the indicated pGAD-Alfy plasmids using a standard lithium acetate method. Selection of transformed yeast was done on DOB-agar plates lacking tryptophan and leucine. A positive interaction was scored by measuring β-galactosidase activities of duplicate transformants, as previously described (Guarente et al., 1982) The data obtained from 4 independent experiments are presented as mean±SEM. Alternatively, freshly transformed colonies were streaked on DOB-agar plates lacking tryptophan, leucine and histidine and were grown for 2-3 days at 30°C.

Proteasome Assay

Proteasome activity was analyzed using the Proteasome-Glo™ Cell-based assay (Promega, Madison, WI), which measures the chymotrypsin-like protease activity associated with the proteasome complex, according to the manufacturers' instruction. Briefly, HeLa cells (5×10^3) were seeded in 96 wells white walled plate on the last day of siRNA transfection. The cells were exposed for 10 min to the Proteasome-Glo™ reagent and the chymotrypsin-like protease activity was measured using the GloMax™ 96 Luminometer (Promega, Madison, WI).

Drosophila Lines and Genetic Crosses

Flies were cultured and maintained on standard cornmeal-molasses-yeast medium and lines obtained from the Bloomington Stock Center (flybase.bio.indiana.edu). The *EP(2)2299* (*UAS-EP-bchs*, *bchs*⁴) allele was from the Röth P-element collection that contains a UAS-P-element construct insertion that allows Gal4-driven expression of the full-length Bchs protein. The *UAS-bchs-C1000* P-element construct was generated from an EcoR1 cDNA fragment containing the C-terminal 1000 amino acids of Bchs, cloned in to the pUAST vector (Brand and Perrimon, 1993), using a linker sequence encoding a start methionine. This region of Bchs/Alfy contains the BEACH, WD40 and FYVE domains. The *UAS-PolyQ127* line was a gift from Dr. Seymour Benzer (California Institute of Technology, CA) and was genetically recombined on to the same chromosome as the *GMR-Gal4* driver. This line was crossed as a single unit with different UAS lines. Crosses were maintained at 25°C and F1 progeny scored for modification of eye pigmentation, size, shape, surface texture and necrosis. Representative digital eye images were taken with a Leica MZ6 dissection microscope and Nikon Coolpix 990 camera system and processed using Adobe Photoshop 7.0 and Canvas 8.0 imaging software.

Statistical Information for Figures

Figure 2G: ANOVA revealed significant difference of ‘siRNA’ ($p<0.0001$; $F(3,84)=11.674$), of ‘Dox treatment’ ($p<0.0001$; $F(1,84)=24.081$) and an interaction between ‘siRNA’ and ‘Dox treatment’ ($p=0.0005$; $F(3,84) = 6.542$) on ‘% cells with aggregates’. Fisher PLSD post hoc comparisons are shown in Figure legends. Cumulative data from $n=4$ experiments.

Figure 3A. HeLa cells: ANOVA revealed a significant effect of treatment (CM vs Starv vs Starv+3-MA) ($F(2,18)=23.961$; $p<0.0001$), no significant effect of siRNA (siCtrl vs siAlfy) on ‘% proteolysis’ ($F(2,18)=0.411$; $p=0.6991$), and no interaction between treatment and siRNA ($F(4,18)=0.322$; $p=0.8591$) on ‘% Proteolysis’.

N2a: ANOVA revealed an effect of treatment ($F(2,24)=18.328$; $p<0.0001$), no significant effect of siRNA ($F(1,24)=0.907$; $p=0.3505$) and no interaction between siRNA and treatment ($F(2,24)= 0.068$; $p=0.9343$) on ‘% proteolysis’.

Figure 3B. ANOVA revealed a significant effect of Treatment (CM vs Starv vs Starv+BafA1) ($F_{(2,18)}=4.538$; $p=0.0254$), no significant effect of siRNA (siCtrl vs siALFY) ($F_{(1,18)}=0.093$; $p=0.7641$) and no significant interaction between Treatment and siRNA ($F_{(2,18)}=0.040$; $p=0.9680$) for ‘LC3 II/LC3 I ratio’.

Figure 3D. ANOVA revealed a significant effect of ‘Starvation’ (CM vs Starv) ($F_{(1,480)}=6128.3$; $p<0.0001$) and siRNA (siCtrl vs siALFY vs siATG7) ($F_{(2,480)}=656.399$; $p<0.0001$) and a significant interaction between ‘Starvation’ and ‘siRNA’ ($F_{(2,480)}=892.097$; $p<0.0001$) on LC3 puncta. Fisher PLSD posthoc comparisons within Starv revealed a significant difference between siCtrl and siATG7 ($p<0.0001$) and between siALFY and siATG7 ($p<0.0001$), but not between siCtrl and siAlfy ($p=0.501$).

Figure 3F. ANOVA revealed a significant effect of Treatment (Fed vs Starv) ($F_{(1,12)}=156.938$; $p<0.0001$) and Genotype (WT vs Bchs mt vs Atg1 mt) ($F_{(2,12)}=26.665$; $p<0.0001$) and an interaction between treatment and Genotype ($F_{(2,12)}=23.450$; $p<0.0001$) for ‘Number of Lysotracker Puncta’. Fisher PLSD posthoc comparisons within Fed found no significant difference between WT and Bchs mt ($p=0.2151$), but found significant difference between WT and Atg1 mt ($p=0.0005$) and between Bchs mt and Atg1 mt ($p=0.0017$).

Figure 6A. ANOVA revealed a significant effect of ‘Alfy transfection’ on ‘Number of aggregates per cell’ ($F_{(3,138)}=14.836$; $p<0.0001$). Posthoc Fisher PLSD analysis found a significant decrease by C-terminus Alfy ($p<0.0001$), but no significant effect of N-terminus Alfy ($p=0.4241$) or middle Alfy ($p=0.3325$).

Figure 6D. ANOVA revealed a significant effect of ‘Genotype’ on ‘Necrotic Patches per Eye’ ($F_{(4,252)}=38.443$; $p<0.0001$). Post hoc Fisher PLSD analysis found a significant decrease of ‘Necrotic Patches per Eye’ in flies that co-express PolyQ127 with C1000Bchs ($p<0.0001$) or in flies that co-express full length Bchs (Bchs) ($p<0.0001$). Fisher PLSD comparisons also reveal a loss of reversal when PolyQ127 is co-expressed with C1000 and dsAtg8a ($p=0.9977$). Expression of dsAtg8a alone in PolyQ127 flies did not significantly impact ‘Necrotic Patches per Eye’ ($p=0.2391$).

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