Huntingtin functions as a scaffold for selective macroautophagy

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Selective macroautophagy is an important protective mechanism against diverse cellular stresses. In contrast to the well-characterized starvation-induced autophagy, the regulation of selective autophagy is largely unknown. Here, we demonstrate that Huntingtin, the Huntington disease gene product, functions as a scaffold protein for selective macroautophagy but it is dispensable for non-selective macroautophagy. In Drosophila, Huntingtin genetically interacts with autophagy pathway components. In mammalian cells, Huntingtin physically interacts with the autophagy cargo receptor p62 to facilitate its association with the integral autophagosome component LC3 and with Lys-63-linked ubiquitin-modified substrates. Maximal activation of selective autophagy during stress is attained by the ability of Huntingtin to bind ULK1, a kinase that initiates autophagy, which releases ULK1 from negative regulation by mTOR. Our data uncover an important physiological function of Huntingtin and provide a missing link in the activation of selective macroautophagy in metazoans.

In macroautophagy (hereafter referred to as autophagy), the material to be degraded (cargo) is first sequestered into a double-membrane vesicle (autophagosome) that subsequently fuses with the lysosome. Contrary to the ‘in bulk’ engulfment of cytosolic material observed on induction of autophagy by starvation, most nutrient-independent stresses (that is, proteotoxicity, lipotoxicity, organelle damage) activate selective autophagy. In selective autophagy only the altered cytosolic components are recognized by cargo adaptors such as p62 (also known as SQSTM1; ref. 2), and then targeted for lysosomal degradation. Despite a growing appreciation of the role of selective autophagy in intracellular quality control and cellular stress response, its regulation and how the autophagy machinery is recruited to the cargo for autophagosome formation are not well defined. In yeast, scaffold proteins such as Atg11 facilitate the effective recruitment of cargos into autophagosome through their interaction with cargo receptors. However, few such scaffolds have been identified in mammals. A second difference between starvation-induced ‘in bulk’ autophagy and selective autophagy is their regulation. Activation of autophagy on starvation is attained through repression of the master nutrient sensor mTOR complex 1 (mTORC1), which promotes release and activation of an autophagy-initiation kinase ULK1. In contrast, selective autophagy can be activated independently of mTORC1 through relatively poorly understood mechanisms.

Huntingtin (Htt), the protein encoded by the gene mutated in Huntington disease, is a large protein with many proposed functions. We previously reported defective autophagy in Huntington disease-affected neurons manifested by the presence of ‘empty autophagosomes’ and suggestive of failure of cargo sequestration. Here, we show that Htt positively modulates selective autophagy but not starvation-induced autophagy in fly and mammalian cells. Htt contributes to cargo recognition by facilitating binding of p62 to ubiquitin (Ub)-K63-modified cargos and to LC3, an integral autophagosome

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Figure 1  *Drosophila* huntingtin interacts genetically with the autophagy pathway. (a) Representative scanning electron micrographs of the external thorax (a1–a4) and phalloidin F-actin staining of the internal muscle structure underlying the thorax (a5–a8) in adult flies of the indicated genotype. Arrows: collapsed thorax. White dotted lines: missing dorsal longitudinal muscles. ATau: ectopic Tau expression from a single copy of the UAS–Tau-ΔC transgene driven by a single copy of the A307–Gal4 line (A307–Gal4 > UAS–Tau-ΔC). Right: quantification of penetrance (top) or muscle loss (bottom; n = 3 independent experiments). (b, c) Phalloidin F-actin staining in fly mutants of the indicated genotypes either homozygous for *atg8aΔd4* or double heterozygous for *dhttko;* and other mutations: *atg8aΔd4*, *atg1ΔC*, *atg13Δko*, *atg7Δko* and Ref(2)P*Δko*. Right: quantification of percentage of muscle loss (n = 4 independent experiments). (d) Representative confocal images (d1–d3) of whole-mount adult brains expressing the mCherry–GFP–Atg8a reporter under a pan-neuronal driver (Appl–Gal4). High-magnification view of outlined areas (d4–d6). (e) Quantification of mCherry–Atg8a-positive puncta in flies of the indicated genotypes (n = 15 fly brains (d1), n = 18 (d2), n = 19 (d3), pooled from 3 independent experiments). (f, g) Representative immunoblot (f) and quantification (g) of Ref(2)P protein from *dhttko–/–* mutant flies and age-matched controls with the indicated genotypes (n = 3 independent experiments). (h, i) Representative immunoblot of Tau-ΔC in fly mutants homozygous for *atg8aΔd4* (h) or *dhttko* (i). (j) Representative immunoblot of samples from flies co-expressing Tau-ΔC together with control LacZ (lanes 2 and 5) or with DTS7 (lanes 3 and 6), a temperature-sensitive dominant-negative mutant allele of a proteasome subunit. Inactivation of the proteasome by raising DTS7-expressing flies at the non-permissive temperature of 28 °C (lanes 4–6) did not cause a higher level of accumulation of Tau-ΔC compared to the control. All values are mean ± s.e.m. and differences are significant for *P < 0.05* using analysis of variance plus Bonferroni’s test. Scale bars, 20 μm for d4–d6 and 100 μm for all others. Uncropped images of blots are shown in Supplementary Fig. 9.

**RESULTS**

*Drosophila* huntingtin genetically interacts with autophagy pathway components

Homoyzogous flies lacking its single *hht* homologue (*dhtt*ko) are fully viable with only mild phenotypes. In a genetic screen for the physiological function of Htt, ectopic expression of a truncated form of the microtubule-binding protein Tau (Tau-ΔC; truncated after Val 382) induced a prominent collapse of the thorax in *dhtt*ko flies due to severe muscle loss below (Fig. 1a7) not observed by Tau expression alone, and accelerated decline in mobility and lifespan. These phenotypes were fully rescued by the *dhtt* genomic rescue transgene (*dhttko*; ref. 13; Fig. 1a and Supplementary Fig. 1a–c), suggesting that *dhtt* protects against Tau-induced pathogenic effects. Although heterozygous *dhttko/+* flies expressing Tau (ATau; *dhttko/+*) seem normal, removing a single copy of the fly LC3 gene, *atg8a* (*atg8aΔd4* mutant)14, in these flies also induced a collapsed thorax and muscle loss, which could be phenocopied by expressing Tau in homozygous *atg8aΔd4–/–* flies alone (Fig. 1b and Supplementary Fig. 1d). Four additional components of the early steps of the autophagy pathway, *atg1* (ULK1), *atg7* and *atg13*, and an adaptor for the selective recognition of autophagic cargo, *Ref(2)P* (p62; ref. 15), also exhibit strong genetic interactions with *dhtt* (Fig. 1c...
Figure 2  Huntingtin is functionally conserved between the fly and humans and is required for effective autophagy in mammals. (a–e) hHtt rescued dhtt−/−-associated phenotypes. (a) Thorax pictures (top) and phalloidin F-actin staining (bottom) of flies. Arrows: collapsed thorax. White dashed lines: muscle loss. Scale bars, 100 μm. (b) Representative confocal images of adult brains from flies expressing GFP–mCherry–Atg8a (scale bar, 100 μm) and staining (bottom) of flies. Arrows: protein aggregates. Scale bar, 10 μm. (c) Quantification. n=12 fly brains (b1), n=10 (b2), n=9 (b3), pooled from 3 independent experiments. (d) Representative Ref(2)P immunoblot in mutants flies of the indicated ages and genotypes. (e) Quantification. n=3 independent experiments. (f) Immunofluorescence for polyubiquitylated proteins. Arrows: protein aggregates. Scale bar, 10 μm. (k,l) LC3-II flux in cells treated as in i; representative immunoblot (k) and quantification (l) of LC3-II steady-state levels (left), net flux (middle) and synthesis (difference between 2 and 4 h; right) (n=6 plates in 6 independent experiments, with triplicate wells per condition). (m) Representative electron micrographs of cells maintained in serum-supplemented media. Bottom: higher-magnification images of the outlined areas; for control cells, top row of insets shows higher-magnification images of the outlined areas and bottom row shows additional examples of autophagic vacuoles from other control cells. (n) Quantification (from left to right) of autophagic vacuole (AV) number per section, relative cytosolic area occupied, percentage containing cargo and number per section containing single (selective) or multiple (in bulk) cytosolic content or an empty lumen (n=12 micrographs for each condition from 3 independent experiments, with 4 micrographs per experiment). (o) Representative images of immunogold labelling for LC3. Insets: outlined areas at higher magnification. Scale bars, 2 μm. All values are mean ± s.e.m. and differences are significant for *P<0.05 using either analysis of variance plus Bonferroni’s test (c,e) or Student's t-test (c,e,l,n). Uncropped images of blots are shown in Supplementary Fig. 9.
and Supplementary Fig. 1e). Consistent with its pivotal role in autophagy initiation, loss of atg1 induced the strongest defect, and Tau expression could induce a mild muscle loss phenotype even in heterozygous null atg1ΔΔΔ (Fig. 1c). Collectively, these genetic interaction studies suggest a role for d htt in autophagy.

Drosophila huntingtin positively regulates autophagy in vivo

Using the mCherry–GFP–Atg8a fusion reporter to directly measure autophagic flux in adult d httko−/− brains, we found similar number of red fluorescent punctae (acidic autolysosomes originating from autophagosome/lysosome fusion) in young mutant and control flies, but the number of punctae was reduced in old d httko−/− brains when compared with age-matched controls (Fig. 1d,e). As we did not observe autophagosome accumulation (co-localized green and red puncta), we concluded that the absence of d htt in older animals is associated with reduced autophagosome formation. The fact that levels of Ref(2)P were significantly higher in old d httko−/− brains compared with brains from age-matched wild-type controls (Fig. 1f,g) suggested a possible preferential compromise in selective autophagy in these animals.

Consistent with the role of basal autophagy in quality control in non-dividing cells, we found that brains from 5-week-old d httko−/− contained almost double the amount of ubiquitylated proteins, a marker of quality control failure, compared with wild-type flies (Supplementary Fig. 2a). As genetic interaction analysis and specific ubiquitin proteasome system (UPS) reporters all failed to reveal a functional link between d htt and the UPS pathway (Supplementary Fig. 2b–f), we propose that the defects in autophagic activity are the main cause of diminished quality control and increased accumulation of ubiquitylated proteins in d httko−/− mutants.

Drosophila huntingtin is required for intracellular quality control

Selective autophagy is induced in response to proteotoxic stress. The truncated Tau−ΔC used in our genetic studies is preferentially degraded through autophagy in cortical neurons, serving as a model of proteotoxicity when ectopically expressed. We confirmed lower stability of Tau−ΔC compared with full-length Tau in wild-type flies (Supplementary Fig. 3a) and in UPS mutants, but found significantly higher levels of Tau−ΔC when expressed in atg8a mutants and in d httko−/− mutant flies (Fig. 1h–j), suggesting that autophagy is essential for the clearance of Tau−ΔC also in flies and that d htt plays a role in this clearance.

In contrast, loss of d htt did not affect flies’ adaptation to nutrient deprivation, which typically induces robust ‘in bulk’ autophagy. Fat bodies of early third instar larvae expressing mCherry–Atg8a, where starvation-induced autophagy can be readily detected, failed to reveal any significant difference between wild-type and d httko−/− flies and they die at the same rate as wild-type flies when tested for starvation resistance (Supplementary Fig. 2g–i). Thus, although d htt is necessary for selective autophagy of toxic proteins such as Tau−ΔC, it is dispensable for starvation-induced autophagy in flies.

Huntingtin’s function is conserved from flies to humans

Expression of human Htt (h htt) in d httko−/− null flies rescued both the mobility and longevity defects of d httko−/− mutants and partially rescued the Tau-induced morphological and behavioural defects of d httko−/− flies (Fig. 2a and Supplementary Fig. 3b–f). h htt also suppressed almost all of the autophagic defects observed in d httko−/−, including decreased levels of autolysosomes, increased levels of Ref(2)P and of total ubiquitylated proteins, and accumulation of ectopically expressed Tau−ΔC (Fig. 2b–e and Supplementary Fig. 3g–i), suggesting that the involvement of d htt in autophagy is functionally conserved. In fact, confluent mouse fibroblasts knocked down for Htt (Htt(−)) exhibited significantly lower basal rates of long-lived proteins’ degradation than control cells, which were no longer evident on chemical inhibition of lysosomal proteolysis or of macroautophagy, thus confirming an autophagic origin of the proteolytic defect (Fig. 2g,h). Htt(−) fibroblasts also exhibited higher p62 levels and accumulate ubiquitin aggregates even in the absence of a proteotoxic challenge (Fig. 2i,j). As in d httko−/− flies, Htt knockdown in mammalian cells did not affect degradation of CL1−GFP (a UPS reporter), β-catenin (a UPS canonical substrate) or proteasome peptidase activities (Supplementary Fig. 4a–c). Reduced autophagic degradation in Htt(−) cells was not due to a primary lysosomal defect, as depletion of Htt did not reduce lysosomal acidification, endolysosomal number (if anything, we observed an expansion of this compartment) or other lysosomal functions such as endocytosis (transferrin internalization shown as an example; Supplementary Fig. 4d–h). In fact, analysis of the lysosomal degradation of LC3-II revealed that autophagic flux and autophagosome formation were preserved and even enhanced in Htt(−) fibroblasts at basal conditions (Fig. 2k,l).

Mammalian Huntingtin is required for selective autophagy

In light of the above observed role of Htt in autophagy under basal conditions, we then analysed the requirement for Htt in autophagy activated in response to different cellular stressors, when only specific cytosolic cargo is degraded. The reduced rates of autophagic degradation of long-lived proteins observed in Htt(−) fibroblasts become even more pronounced on exposure to proteotoxic stress (inhibition of proteasome degradation), lipotoxic stress (oleic challenge) or the mitochondrial depolarizing agent FCCP (Fig. 3a). However, on starvation (serum removal or incubation in nutrient-free media), control and Htt(−) fibroblasts exhibited a similar increase...
Figure 3 Huntingtin functions in selective macroautophagy. (a) Htt loss reduced autophagy-dependent degradation in response to different stressors. Long half-life protein degradation rates in NIH3T3 cells control (Ctrl) or knocked down for Htt (Htt(−)) cultured in serum-deprived (−) or -supplemented (+) medium without additions or with the indicated stressors (n=6 plates in 3 independent experiments, with triplicate wells per condition). (b,c) GST–BHMT assay. Representative immunoblot of the time course of Tau-ΔC levels in HeLa cells (d) and quantification of Tau-ΔC protein (e) (n=3 independent experiments). (f–i) Htt knockdown in NIH3T3 fibroblasts reduces lipophagy. (f) Higher susceptibility of Htt(−) cells to lipotoxicity (oleic or palmitic acid) but not to genotoxicity (etoposide) (n=6 plates in 3 independent experiments, with triplicate wells per condition). (g,h) Accumulation of lipid droplets (LD) in oleic-treated cells. Representative images of Bodipy493/503-stained cells (g) and quantification of LD number per cell and average LD area (h). Scale bar, 10 μm (n=3 independent experiments where a total of 100 cells were analysed per condition). (i) Reduced increase in oxygen consumption rates (OCR) on oleic (OL) challenges (n=6 plates in 4 independent experiments, with 4 wells per condition). (j) Reduced β-oxidation measured as release of [14C]carbon dioxide in [14C]oleate-loaded cells (n=4). (k,l) LC3 immunostaining in BODIPY493/503-stained cells. (k) Representative images (insets: higher-magnification images). Arrows: depolarized mitochondria. Scale bar, 10 μm. (l) Quantification: percentage of depolarized mitochondria (MitoTracker-positive and MitoTracker Red CMXROS-negative). (m–p) Htt knockdown in NIH3T3 fibroblasts reduces mitophagy. (m,n) Htt(−) cells have a higher content of depolarized mitochondria (MitoTracker-positive and MitoTracker Red CMXROS-negative). (m) Representative images (insets: higher magnification images). Arrows: depolarized mitochondria. Scale bar, 10 μm. (n) Quantification: percentage of depolarized mitochondria (n=3 independent experiments; a total of 85 cells were analysed per condition). (o,p) LC3 immunostaining in MitoTracker-stained cells. (o) Representative images in both channels and co-localization pixels in white (insets: higher-magnification images). Scale bar, 10 μm. (p) Quantification of LC3/MitoTracker co-localization (n=3 independent experiments where a total of 110 cells were analysed per condition). In all studies with lentivirus-mediated shRNA (f-p) control cells were transduced with viral particles carrying the empty vector. All values are mean ± s.e.m. and differences are significant for *P<0.05 using Student’s t-test (a,f–j,l,n,p) or analysis of variance plus Bonferroni’s test (e). Uncropped images of blots are shown in Supplementary Fig. 9.
Figure 4 Ultrastructure of cells knocked down for Huntingtin on induction of selective types of autophagy. (a–c) NIH3T3 fibroblasts control (Ctrl) or knocked down for Htt (Het(−)) were exposed to lactacystin (a), oleic acid (b) or the mitochondria-depolarizing agent FCCP (c) and processed for electron microscopy. Images show representative cellular areas and insets show examples of autophagic vacuoles (AV) at higher magnification to appreciate the cargo content. Red arrows: protein aggregates inside AV (a), AV containing lipid material (b) or AV containing mitochondria (c). Yellow arrows: protein aggregates free in the cytoplasm. LD, lipid droplets. Note that induction of proteotoxicity with lactacystin favours autophagic sequestration of proteinaceous aggregate material over sequestration of LD that remain intact under these conditions (a), whereas induction of lipophagy with oleic results in a higher content of AV in close proximity of the LD (b). LD are also preserved from autophagic sequestration in the case of FCCP treatment, where membranous structures compatible with mitochondria undergoing degradation are detected in AV (c). Quantification of the number, average size and percentage of cytosolic area covered by AV is shown on the right (a) or at the bottom (b,c). n = 9 (in a), 9 (in b) and 6 (in c) micrographs pooled from 3 independent experiments. Scale bars, 0.5 μm. All values are mean ± s.e.m. and differences are significant for *P < 0.05 using Student’s t-test.

In long-lived proteins’ degradation and in fragmentation of GST–BHMT, a cargo-based end-point assay for autophagy21 (Fig. 3a,b). In contrast, accumulation of fragmented GST–BHMT was almost abolished in Het(−) fibroblasts subjected to proteotoxic stress by proteasome inhibition (Fig. 3c).

To further analyse the involvement of Htt in selective autophagy, we directly tracked the cargo targeted in each of these stress conditions in stably transfected HeLa cells with inducible expression of Tau-ΔC, degradation of Tau-ΔC was mostly dependent on autophagy (sensitive to 3-methyladenine or Atg7 knockdown but insensitive to proteasome inhibition; Supplementary Fig. 4i–k). Htt knockdown markedly blocked Tau-ΔC turnover (Fig. 3d,e), indicating that, as in flies, Htt is required for the autophagy-mediated degradation of Tau-ΔC.
We next induced selective degradation of lipid droplets (lipophagy) by cell loading with oleic acid\(^{22,23}\). Htt(–) fibroblasts exhibited a higher sensitivity to lipid challenges and a more pronounced accumulation of lipid droplets (Fig. 3f–h). Lipid accumulation in Htt(–) fibroblasts was mostly due to their reduced ability to mobilize and break down intracellular lipid stores, as they failed to increase mitochondrial respiration in response to the lipogenic challenge and exhibited lower rates of lipid β-oxidation (Fig. 3i,j). Immunostaining revealed significantly lower co-localization of LC3 with Bodipy-labelled lipid droplets in Htt(–) cells (Fig. 3k,l), further supporting the idea that Htt is required for selective lipophagy. Similarly, Htt(–) fibroblasts showed a comparatively higher content of depolarized mitochondria (labelled with MitoTracker but negative for MitoTracker Red CMXRos staining) and less co-localization between LC3 and MitoTracker-highlighted mitochondria than control cells, in support of reduced mitophagy (Fig. 3m–p).

Ultrastructural analysis of control and Htt(–) cells on exposure to these three different stressors confirmed the fluorescence data (Fig. 4a–c). Thus, the most abundant fraction of autophagosomes in control cells treated with a proteasome inhibitor were those containing aggregate proteinaceous material, whereas in Htt(–) cells most of the observed autophagic vacuoles had a clear lumen and aggregates were instead detectable free in the cytosol (Fig. 4a). Similarly, treatment with oleic acid or FCCP increased the fraction of autophagic vacuoles containing lipids or mitochondria, respectively, in control cells whereas this switch towards a specific autophagic cargo was not observed in Htt(–) cells (Fig. 4b–c). Interestingly, in contrast to the higher autophagic vacuole content observed in Htt(–) fibroblasts under basal conditions (Fig. 2m,n), the number and cell fraction occupied by autophagic vacuoles on exposure to any of these stressors were significantly lower in Htt(–) than in control cells (Fig. 4a–c), in support of a failure to maximally induce selective autophagy in Htt-deficient cells.

Complete ablation of Htt (mouse embryonic fibroblasts (MEFs) from mice knocked out for Htt (ref. 24) or Htt knockdown in other cell types (neuroblastoma N2a cells and mouse striatal cells), also led to higher levels of p62 and reduced co-localization of LC3 with markers of lipid droplets or mitochondria on exposure to oleic acid or FCCP, respectively (Fig. 5). Overall, our results support the hypothesis that Htt is dispensable for starvation-induced autophagy but essential for at least three different types of selective autophagy, aggrephagy of either a specific protein (Tau) or a pool of aggregate proteins (proteasome inhibition), lipophagy and mitophagy.

Further analysis of changes in autophagic flux in the absence of Htt in different cell types under different experimental conditions revealed that, in contrast to the increase in basal autophagosome formation and autophagic flux observed in Htt(–) fibroblasts (Fig. 2k,l), Htt depletion in HeLa cells had indiscernible effects on basal LC3-II flux and on GST–BHMT fragmentation under basal conditions (Supplementary Fig. 5a,b). These data probably reflect the lower...
Figure 6  Huntingtin modulates autophagic induction and physically interacts with p62 and ULK1 proteins through two non-overlapping conserved regions. (a) Representative immunoblot of LC3 in HEK293T cells treated as indicated. Bottom: quantification of LC3-II levels and net LC3-II flux (lateral numbers shown net differences in LC3-II levels on addition of BafA1) normalized against loading control actin (n = 3 independent experiments). (b) Immunostaining for LC3 in HeLa cells treated as indicated. Scale bar, 10 μm. (c,d) Quantification of LC3-positive puncta plotted by different inhibitor treatments (c) or by Htt genotype (d). Note that although the ratio of LC3 in cells treated or not with BafA1 is comparable in control and Htt(–) cells, the net changes in LC3 puncta content on addition of BafA1 (shown in the lateral numbers) are markedly lower in these cells (n = 5 wells, pooled from 3 independent experiments, >150 cells per experiment). (e) Representative immunoblot to show co-immunoprecipitation experiments using whole-animal extract from dhttko–/– as a negative control or a genome-tagging MiMIC fly line expressing 3 × HA-tagged dHtt (n = 3 independent experiments). (f,g) Representative immunoblot to show co-immunoprecipitation experiments between Htt and p62 or ULK1 in HEK293T cell lines (f) and MEF cells (g) in comparison with negative controls, Htt-siRNA-treated (f) or Htt-KO MEF (g) cells. Input lines show the Triton X-100-soluble fraction from the whole-cell lysate used for co-immunoprecipitation on normalization of soluble p62 levels across samples (n = 3 independent experiments). (h) Representative confocal images of immunofluorescent stained cells for Htt (green), p62 (red) and Draq5 (blue). Bottom: high-magnification view of outlined areas. Scale bar, 5 μm (top) or 2 μm (bottom) (n = 3 independent experiments). (i-k) Mapping of p62- and ULK1-interacting regions in Htt. (i) Schematics of fly and human conserved regions (blue) in Htt (top) and of the Htt deletions (green) generated in this study (bottom). (j,k) Representative immunoblots for co-immunoprecipitation assays using the HA-tagged Htt deletions to pull down Myc-p62 (j) or Myc–ULK1 (k). Whole-cell extracts (WCE) are shown in the right. Myc–p62 was pulled down by the C-terminal CD and D6 fragments in Htt and of the Htt deletions (green) generated in this study (bottom). (i-k) Representative immunoblots for co-immunoprecipitation assays using the HA-tagged Htt deletions to pull down Myc-p62 (j) or Myc–ULK1 (k). Whole-cell extracts (WCE) are shown in the right. Myc–p62 was pulled down by the C-terminal CD and D6 fragments in Htt and of the Htt deletions (green) generated in this study (bottom). (i-k) Representative immunoblots for co-immunoprecipitation assays using the HA-tagged Htt deletions to pull down Myc-p62 (j) or Myc–ULK1 (k). Whole-cell extracts (WCE) are shown in the right. Myc–p62 was pulled down by the C-terminal CD and D6 fragments in Htt and of the Htt deletions (green) generated in this study (bottom).
dependence on Htt-mediated selective autophagy in these rapidly dividing cells, where quality control is less important. However, when we forced activation of quality control selective autophagy in these cells by inflicting proteotoxic stress with the proteasome inhibitor, Htt knockdown markedly blocked the induction of autophagy visible in the control cells as an increase in steady-state levels and clearance of LC3-II (Fig. 6a). Immunostaining for LC3 confirmed a reduced increase in the number of LC3-positive puncta in the Htt(−) HeLa cells on proteotoxic stress (Fig. 6b,c). Transfection with GFP-Htt restored the defective autophagic flux during proteotoxic stress both in Htt(−) HeLa cells and in Htt-KO MEFs, but did not affect autophagy activation in response to serum withdrawal (Supplementary Fig. 5c,d). Blockage of lysosomal proteolysis with bafilomycin-A1 in Htt(−) cells exposed to proteasome inhibition still increased the number of LC3-positive puncta (Fig. 6b,d), suggesting that, in agreement with the electron microscopy data (Fig. 4), autophagosome clearance is normal in Htt-depleted cells and that their reduced LC3 flux during proteotoxic stress is mainly due to reduced autophagosome formation. Consistently, double labelling with the lysosome marker LAMP1 revealed lysosomal accumulation of LC3 in bafilomycin-A1-treated cells in both control and Htt(−) cells, indicating that fusion of autophagosomes with lysosomes still occurs in the absence of Htt (Supplementary Fig. 5e). In contrast, Htt knockdown did not significantly affect the levels or processing of LC3-II on induction of non-selective autophagy by nutrient depletion (Supplementary Fig. 5f). We found similar defects in induction of selective autophagy on exposure to proteasome inhibitors, oleic acid or FCCP on Htt depletion in mouse fibroblasts, MEFs, striatal and neuroblastoma cells expressing the mCherry–GFP–LC3 fusion reporter, whereas their ability to upregulate autophagic flux in response to nutritional deprivation remained unaffected (Supplementary Fig. 5g–k). These results support the idea that Htt is important in selective autophagy for both initiation of autophagosome formation and cargo recognition.

**Huntingtin physically interacts with p62 and ULK1 proteins**

To explore how Htt is involved in autophagy regulation, we next examined potential physical interactions between Htt and the autophagy components identified in our fly-based genetic screens (Fig. 1). Using whole-animal extracts from a genome-tagging fly line expressing 3×HA-tagged dHtt, we found that dHtt strongly co-immunoprecipitated with endogenous Ref(2)P (Fig. 6e). Experiments in mammalian cells confirmed co-immunoprecipitation of both endogenous and tagged Htt and p62, as well as between Htt and ULK1, the mammalian Atg1 homologue
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(Fig. 6f and Supplementary Fig. 6a–d). Notably, we previously reported that p62 co-localized and interacted with mutant Htt in autophagosomes
32
. Hence, results from the fly and mammalian systems together support the idea that p62 and ULK1 are conserved binding partners of Htt.

Intriguingly, the amount of endogenous p62 that co-immunoprecipitated with endogenous Htt was significantly increased on induction of proteotoxic stress (proteasome inhibitor treatment), whereas the level of co-immunoprecipitated ULK1 remained similar (Fig. 6g). Immunofluorescence for endogenous Htt and p62 in HeLa cells also revealed enrichment of Htt in the large p62-positive bodies (sequestosomes)
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 that form around the nucleus on proteasome inhibition (Fig. 6h), suggesting a translationaltranslocation of Htt to the p62 bodies in response to autophagy induction. Tagged ULK1 has been shown to co-localize with p62 (ref. 27), raising the possibility that Htt might form a tertiary complex with p62 and ULK1.

To map the regions responsible for the association of Htt with p62 and ULK1, we generated Htt deletions and through co-immunoprecipitation identified two non-overlapping conserved regions
1,2,28
 in Htt, the carboxy-terminal D6 and the middle D3 regions that bind to p62 and ULK1, respectively (Fig. 6i–k and Supplementary Fig. 6e–g).

**Huntingtin facilitates p62-mediated cargo recognition efficiency**

Given the defective sequestration of selective cargo inside autophagosomes in Htt(−) cells and the direct interaction between Htt and p62, we investigated whether Htt functions with p62 in cargo recognition. p62 is involved in selective autophagy by simultaneous binding with LC3 and ubiquitylated cargos for their subsequent engulfment by the forming autophagic membrane
2. Cargo recognition by p62 initiates with the formation of sequestosomes (detergent-resistant p62 bodies)
1,26. Accordingly, treatment with proteasome inhibitors gradually decreased soluble p62 and increased insoluble p62 in control cells. However, p62 redistribution and formation of large p62 bodies were no longer observed on depletion of Htt (Fig. 7a–c and Supplementary Fig. 7a–c; depletion of Beclin 1, essential for autophagosome formation
1 is shown as a positive control). In contrast, the fraction of cellular p62 degraded by autophagy during starvation did not change on Htt knockdown (Supplementary Fig. 7d).

Three functional domains in p62 coordinate its roles in selective autophagy: the self-polymerization (PB1) and ubiquitin-binding (UBA) domains are critical for p62 body formation
2, and the middle motif (LIR) for its interaction with LC3 for cargo-based autophagic degradation
29. We found that knockdown of Htt had no obvious effect on p62 polymerization (HA–p62 and Myc–p62 interaction) or its interaction with ubiquitylated proteins containing the Lys 48 linkage-specific modification (Ub-K48), but strikingly reduced the amount of p62 that co-immunoprecipitated with ubiquitylated substrates containing the Lys 63 linkage-specific modification
30. (Ub-K63; Supplementary Fig. 7e–g). These findings were also reproduced using transfected HA-tagged Ub-K63 and Ub-K48 and pulldowns with anti-HA (to discard nonspecific antibody effects; Supplementary Fig. 7h–i) and in Htt-KO MEFs (to discard nonspecific siRNA-mediated effects; Fig. 7d,e). The above results raise the possibility that Tau-ΔC is also a target of Ub-K63 modification. Indeed, we found that Tau-ΔC protein was preferentially modified by Ub-K63 but not Ub-K48 in HEK293T cells (Fig. 7f). Co-immunoprecipitation experiments showed that p62 physically interacts with Tau-ΔC and their affinity was compromised after Htt knockdown (Fig. 7g). Overall, these findings indicate that Htt facilitates p62 binding to Ub-K63-modified proteins that are preferentially degraded by selective autophagy
31.

Last, we investigated the effect of Htt knockdown in the interaction of p62 with LC3. The ability of LC3 to co-immunoprecipitate endogenous p62 was markedly reduced in Htt(−) cells and, accordingly, co-localization of p62 and LC3 was also significantly...
Huntingtin facilitates p62-mediated cargo recognition efficiency.

Figure 7 Huntingtin facilitates p62-mediated cargo recognition efficiency.
(a) Representative immunoblot of Triton X-100-soluble and -insoluble p62 in HEK293T cells treated as indicated (n=3 independent experiments). (b) Confocal images of HeLa cells immunostained for endogenous p62 (green) and Drq5 (blue). Scale bar, 8 μm. (c) Quantification of large p62 bodies. n=3 independent experiments >150 cells. (d,e) Representative immunoblots of endogenous proteins with Ub-K63 or Ub-K48 modifications immunoprecipitated in MEF cells from wild-type (WT) or Htt knockout (Htt-KO) mice using anti-Ub-K63 (d) or anti-Ub-K48 (e) antibodies. Co-immunoprecipitated p62 is also shown. (f) HA–ΔC–GFP and HA-tagged Ub-K63 or Ub-K48 were co-transfected into HEK293T cells, followed by an in vitro ubiquitination assay with anti-GFP antibody and probing with anti-HA antibody. (g) Representative immunoblots of endogenous p62 co-immunoprecipitated with an anti-GFP antibody in stable Tau–ΔC–GFP-expressing HeLa cells, control or treated with siRNA against Htt (n=3 independent experiments). (h–j) Htt knockdown compromised p62/LC3 interaction in NIH3T3 fibroblasts. (h) Representative immunoblot of co-immunoprecipitation experiments in NIH3T3 cells for LC3. Inp: input, IP: immunoprecipitated, FT: flow-through. (i,j) Co-immunostaining for p62 and LC3 in confluent NIH3T3 cells. (i) Representative images of merged channels of full fields (top) and high-magnification of outlined areas (bottom). Scale bars, 10 μm. (j) Quantification: percentage of co-localization (n=4 independent experiments; a total of 80 cells were analysed per condition). All values are mean ± s.e.m. and differences are significant for *P<0.05 using analysis of either variance plus Bonferroni’s test (c) or Student’s test (j). Uncropped images of blots are shown in Supplementary Fig. 9.

reduced on Htt loss (Fig. 7h–j). Although it is not possible to discriminate whether the reduced p62 and LC3 interaction is primarily due to the loss of Htt or secondary to the inability of p62 to bind K63-ubiquitylated cargo, these findings uncover an essential role for Htt in p62-dependent cargo recognition required for proper sequestration of specific cargo into autophagosomes during selective autophagy.

Huntingtin–ULK1 and mTOR–ULK1 complexes are mutually exclusive

To investigate the basis for the observed defective autophagosome biogenesis in Htt(−) cells in response to different stressors, we followed up on the physical (Fig. 6f) and genetic (Fig. 1c) interaction of Htt with ULK1, the kinase essential for initiation of both selective and non-selective autophagy. In control cells, both starvation and proteotoxicity (proteasome inhibition) significantly boosted ULK1 kinase activity, measured as increased phosphorylation of myelin-binding protein (MBP), a pseudo-substrate for ULK1 (ref. 25), but Htt knockdown only attenuated proteasome inhibition-mediated ULK1 activation (Fig. 8a). These results suggest that Htt could contribute to the regulation of the initiation of selective autophagy by modulating ULK1 activation while not affecting starvation-induced non-selective autophagy. Consistent with this hypothesis, Htt has no significant impact on AMPK, which positively regulates ULK1 kinase activity in response to energy depletion, presumably by interacting and phosphorylating ULK1 (refs 32–34). Htt knockdown did not disrupt the interaction between AMPK and ULK1, nor did it interfere with AMPK-mediated ULK1 phosphorylation at Ser 555 (Supplementary...
Figure 8 Huntingtin–ULK1 and mTOR–ULK1 complexes are mutually exclusive. (a) Representative autoradiograph to show in vitro ULK1 kinase activity monitored by the phosphorylation level of myelin-binding protein (p-MBP). Equal input of ULK1 protein was verified by immunoblot (n = 3 independent experiments). (b) Representative immunoblot of co-immunoprecipitation experiments using anti-FLAG antibody in HEK293T cells co-transfected with FLAG-tagged ULK1 and Myc-tagged Htt, Raptor or mTOR, as indicated (n = 3 independent experiments). (c,d) Representative immunoblot of co-immunoprecipitation experiments using anti-FLAG (c) or anti-Myc (d) in HEK293T cells co-transfected with tagged Htt, ULK1, mTOR and Raptor, as indicated. (e,f) Representative immunoblots of endogenous co-immunoprecipitation experiments in MEFs using anti-ULK1 or anti-Myc antibodies (f). (g) Representative immunoblot to show reciprocal co-immunoprecipitation experiments in MEFs using anti-ULK1 or anti-mTOR antibodies, as indicated. (h) Representative immunoblot to show in vitro ULK1 kinase assay in HEK293T cells treated as indicated. (i) Representative immunoblot of co-immunoprecipitation assays using anti-FLAG in HEK293T cells transfected with FLAG–ULK1 and HA–Htt as indicated to analyse the association between ULK1 and endogenous Raptor or mTOR proteins (n = 3 independent experiments). (j) A schematic model of Htt regulation of selective autophagy. Htt serves as a scaffolding for selective autophagy by bringing together cargo bound through p62 and the initiator of autophagy ULK1. Basal autophagy: under basal conditions the absence of Htt leads to reduced selectivity in cargo recognition required for quality control autophagy, but it does not affect autophagy induction/autophagosome biogenesis, because basal ULK1 is sufficient to sustain ‘in bulk’ autophagy and basal quality control autophagy. Induced autophagy: maximal activation of autophagy in response to stress requires the release of mTORC1 inhibition over ULK1. In starvation-induced autophagy, inactivation of mTORC1 promotes release and activation of ULK1. Selective autophagy induced in response to different stressors requires Htt to actively compete away ULK1 from the mTORC1 inhibitory complex. Uncropped images of blots/gels are shown in Supplementary Fig. 9.

Fig. 8a,b), two mechanisms of regulation that are critical for AMPK-induced ULK1 activation.35

To determine how Htt specifically activates selective autophagy but not non-selective autophagy when they both share common regulators including ULK1, we systematically examined several established mechanisms governing ULK1 kinase activity. Maximal activation of ULK1 is attained by forming a stable quaternary complex with FIP200, Atg13 and Atg101 (ref. 25). Pulldown assays revealed that Htt did not affect the stability of this complex (Supplementary Fig. 8c). ULK1 is also regulated by the central nutrient sensor mTORC1, which binds to ULK1 and represses its kinase activity to prevent autophagy initiation. Inactivation of mTORC1 during starvation triggers release of active ULK1 from this complex and initiation of non-selective autophagy, whereas the mechanisms that counterbalance the inhibitory effect of mTORC1 on ULK1 for activation of selective autophagy remain poorly understood. We first tested whether Htt affected the kinase activity of mTOR and consequently its inhibitory effect on ULK1. Neither Htt knockdown nor Htt overexpression showed any obvious
effect on mTOR kinase activity when assessed by the phosphorylation of the downstream mTOR effector S6K at Thr 389 (ref. (36) under basal conditions or on activation of mTOR by the upstream regulator Rheb (Supplementary Fig. 8d,e)). This result was further confirmed by an in vitro kinase assay using GST-4E-BP1, a substrate readily phosphorylated by mTOR at Ser 65 after mTOR activation. Ser65–GST–4E-BP1 phosphorylation was unaffected by Htt knockdown (Supplementary Fig. 8f). Thus, Htt might not activate ULK1 by directly inhibiting mTOR activity.

Excitingly, although ULK1 co-immunoprecipitated with Htt, mTOR and Raptor, an adaptor protein critical for mTORC1 activity (Fig. 8b), Htt–ULK1 and mTOR–Raptor–ULK1 exist as two separate mutually exclusive complexes. Specifically, co-immunoprecipitation assays showed that Htt could pull down ULK1 but not mTOR or Raptor (Fig. 8c), and both mTOR and Raptor co-immunoprecipitated ULK1 but not Htt (Fig. 8d). Similar physical interactions were observed for endogenous Htt, ULK1 and mTOR proteins in MEFs (Fig. 8e,f; Htt-KO and ULK1-KO (ref. (37) as controls) and neuroblastoma N2a cells (Supplementary Fig. 8g–i), thus confirming the existence of two mutually exclusive ULK1–Htt and ULK1–mTOR complexes. This mutual exclusion indicates that Htt may promote ULK1 activation during selective autophagy by directly competing for the inhibitory binding of mTORC1 to ULK1. Indeed, under all three different stress conditions, we observed an increased interaction between ULK1 and Htt at the expense of mTOR, and Htt depletion largely abolished this competitive effect (Fig. 8g and Supplementary Fig. 8j,k). Consistently, overexpression of Htt significantly blocked mTOR-mediated repression of ULK1. Basal ULK1-dependent phosphorylation of MBP can be detected in the kinase assay under rich nutritional conditions but not with the kinase-dead ULK1 mutant (FLAG–ULK1-KD; Fig. 8a). This basal ULK1 activity is sensitive to regulation by mTOR, as overexpression of Myc–mTOR substantially repressed MBP phosphorylation and overexpression of a kinase-dead Myc–mTOR mutant showed the opposite effect, probably through a dominant-negative mechanism (Fig. 8h). Interestingly, overexpression of Htt significantly relieved this mTOR-mediated repression of basal ULK1 kinase activity (Fig. 8h), suggesting that Htt antagonizes the negative regulation of mTOR on ULK1 and promotes activation of selective autophagy even in the presence of active mTOR (rich media) by directly competing away ULK1 from the mTORC1–ULK1 complex. Indeed, co-immunoprecipitation assays revealed that Htt overexpression markedly reduced the amount of endogenous mTOR and Raptor proteins associated with ULK1 (Fig. 8i), providing further support for this competition model. Changes in Htt protein levels may also contribute to modulate Htt function on autophagy under physiological conditions, because after exposure to stressors that induce selective autophagy but not in response to starvation, we found modest elevations of endogenous Htt levels in fibroblasts and neuroblastoma N2a cells (Supplementary Fig. 8j,l,m).

DISCUSSION

Selective autophagy is important for intracellular quality control and the cellular stress response but its regulation and how the autophagy machinery is recruited to the cargo for autophagosome formation are still poorly defined processes. In this work, we show that Htt is a scaffold protein that promotes selective autophagy through its ability to simultaneously interact with components involved in two major autophagy steps, p62 and ULK1, and thus modulate both cargo recognition efficiency and autophagosome initiation. First, Htt releases the inhibitory effect of mTOR over ULK1, by directly competing away this kinase from the mTORC1 complex. Second, binding of Htt to p62 enhances the association between this cargo receptor and its Ub-K63-modified substrates. We propose a scaffolding role for Htt in selective autophagy whereby the ability of Htt to simultaneously bind ULK1 and p62 assures the spatial proximity between cargo recognition and autophagy-initiation components (Fig. 8).

We found that Htt is required for at least three types of selective autophagy (aggrephagy, lipophagy and mitophagy) but is dispensable for starvation-induced autophagy. Selective recognition of cargo requires receptors including p62, and Htt facilitates p62-mediated cargo recognition, at least in part, by enhancing the affinity between p62 and Ub-K63-modified cargos and LC3. Selective formation of an autophagosome around the p62-recognized cargo has been attributed to the ability of p62 to simultaneously bind cargo and LC3. However, autophagy initiation requires events that precede lipid conjugation of LC3, such as recruitment to the sites of autophagosome formation of ULK1, one of the earliest autophagy effectors. We propose that Htt directs ULK1 to these sites by forming an Htt–ULK1 complex distinct from the mTOR–ULK1 complex. Of note, both Htt and mTOR contain HEAT repeats, which could be the basis of their mutually exclusive interaction with ULK1 and of the ability of Htt to compete with mTOR for ULK1 binding to achieve maximal activation of selective autophagy. Under basal conditions, binding of Htt to the pool of constitutively active ULK1 identified in this study might be sufficient for preserving basal autophagy-dependent intracellular quality control, potentially by competing away and shielding ULK1 from the inhibitory mTORC1 complex. How this competing activity of Htt is repressed under basal conditions requires future investigation, but it is possible that changes in Htt levels, subcellular location or its myriad of post-translational modifications could contribute to regulate the function of Htt to fine-tune the proper cellular autophagic response against a variety of cellular stresses.

Notably, animals deficient for Htt, ULK1 and p62 show distinctive phenotypes. Htt-KO mice die at embryonic day E7.5 (refs 48–50), whereas p62 and ULK1-KO are viable. These differences are probably partially due to the additional roles each protein plays besides autophagy. For example, the earlier lethality of the Htt-KO mice has been primarily attributed to the essential role of Htt in extraembryonic tissues. Notably, animals deficient for Htt, ULK1 and p62 show distinctive phenotypes. Htt-KO mice die at embryonic day E7.5 (refs 48–50), whereas p62 and ULK1-KO are viable. These differences are probably partially due to the additional roles each protein plays besides autophagy. For example, the earlier lethality of the Htt-KO mice has been primarily attributed to the essential role of Htt in extraembryonic tissues. Furthermore, only one Htt gene exists in both the fly and most vertebrate genomes, whereas ULK1 and ULK2 seem capable of compensating for each other and the function of p62 in autophagic cargo recognition shows some redundancy with other receptors such as NBR1. Interestingly, p62-KO mice exhibit adult-onset neurodegeneration with elevated level of K63-ubiquitylated Tau in the brain and similar, reduced Htt expression or Htt postnatal deletion both result in brain degeneration.

Connections of Htt with autophagy have previously been described and we also reported autophagy abnormalities (‘empty autophagosomes’ phenotype) in the context of Huntington disease. As deleting the polyQ tract in Htt enhances neuronal autophagic activity and longevity in mice, it is attractive to propose that polyQ expansion compromises the role of Htt in selective autophagy.
METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Y-N.R. and Z.X. designed and performed most Drosophila studies and most of the studies on starvation- and MG132-induced autophagy response and Tau-ΔC degradation in mammalian cells; B.P. designed and performed part of the studies on autophagic flux in mammalian cells, and most of the studies of lipophagy and mitophagy, some of the co-immunoprecipitation studies and all the electron microscopy studies and morphometric analysis; Z.C., D.C., A.T., E.E.S. and Y.S. contributed to part of these studies; G.D. performed larva starvation, LysoTracker staining and LC3 reporter assay; G.D. and H.J.B. designed experiments, analysed data and contributed to part of the writing and revision of the manuscript; A.M.C. coordinated the study, designed experiments, analysed data and contributed to the writing and revision of the manuscript. S.Z. coordinated the study, designed experiments, analysed data and contributed to the main writing and revision of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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52. Rodriguez, A. et al. Mature-onset obesity and insulin resistance in mice deficient in the signaling adapter p62. Cell Metab. 3, 211–222 (2006).
53. Cheong, H., Lindsten, T., Wu, J., Lu, C. & Thompson, C. B. Ammonia-induced autophagy is independent of ULK1/ULK2 kinases. Proc. Natl Acad. Sci. USA 108, 11121–11126 (2011).
54. Lee, E. J. & Tournier, C. The requirement of uncoordinated 51-like kinase 1 (ULK1) and ULK2 in the regulation of autophagy. Autophagy 7, 689–695 (2011).
55. Reiner, A., Dragatis, I., Zeitlin, S. & Goldowitz, D. Wild-type huntingtin plays a role in brain development and neuronal survival. Mol. Neurobiol. 28, 259–276 (2003).
56. Dragatis, I., Efstratiadis, A. & Zeitlin, S. Mouse mutant embryos lacking huntingtin are rescued from lethality by wild-type extraembryonic tissues. Development 125, 1529–1539 (1998).
57. Cheong, H. et al. Analysis of a lung defect in autophagy-deficient mouse strains. Autophagy 10, 45–56 (2014).
58. Ramesh Babu, J. et al. Genetic inactivation of p62 leads to accumulation of hyperphosphorylated tau and neurodegeneration. J. Neurochem. 106, 107–120 (2008).
59. Babu, J. R., Geetha, T. & Wooten, M. W. Sequestosome 1/p62 shuttles polyubiquitinated tau for proteasomal degradation. J. Neurochem. 94, 192–203 (2005).
60. Dragatis, I., Levine, M. S. & Zeitlin, S. Inactivation of Hdh in the brain and testes results in progressive neurodegeneration and sterility in mice. Nat. Genet. 26, 300–306 (2000).
61. White, J. K. et al. Huntingtin is required for neurogenesis and is not impaired by the Huntington’s disease CAG expansion. Nat. Genet. 17, 404–410 (1997).
62. Kegel, K. B. et al. Huntingtin expression stimulates endosomal-lysosomal activity, endosome tubulation, and autophagy. J. Neurosci. 20, 7268–7278 (2000).
63. Steffan, J. S. Does Huntingtin play a role in selective macroautophagy? Cell Cycle 9, 3401–3413 (2010).
64. Wong, Y. C. & Holzbaur, E. L. The regulation of autophagosome dynamics by huntingtin and HAP1 is disrupted by expression of mutant huntingtin, leading to defective cargo degradation. J. Neurosci. 34, 1293–1305 (2014).
65. Zheng, S. et al. Deletion of the huntingtin polyglutamine stretch enhances neuronal autophagy and longevity in mice. PLoS Genet. 6, e1000838 (2010).
METHODS

Fly stocks. Flies were maintained on standard Drosophila medium at 25°C unless otherwise indicated. The following fly stocks were obtained from Bloomington Stock Center: A307-Gal4 (no. 6488), Appl-Gal4 (no. 32040), arm-Gal4 (no. 1560), Elav-Gal4 (no. 458), GMR-Gal4 (no. 1104), Cg-Gal4 (no. 7011), UAS-DTSS (no. 6769), UAS-d htt (no. 6785), UAS-mCD8-GFP (no. 5137), UAS-Htt (no. 33810), UAS-LacZ (no. 1114), UAS-GFP-mCherry-Atg8a (no. 37749). The Ref(2)P [3010] line was a hypomorphic insertion allele from the Harvard Medical School (Exelixis Stock Collection). The d htt [13] mutant and d htt-mingine Rescue transgenic (d htt[dcr]) lines were described previously [31]. The UAS--Tau-GFP line was a gift from M. Packard and atg6d, atg8d, atg10d and atg17d are deletional loss-of-function alleles (gifts from T. Neufeld and references therein) [32]. The UAS-GFP-CLC was a gift from U. Pandey [33]. The HA--d htt tagging line was derived from a MIMIC insertional line in the d htt (gene) (see below). The UAS--Tau--FL-GFP was generated by injecting pUAST--Tau--FL--GFP into w1118 embryos together with pr25.7wec helper plasmid followed by standard transgenic procedures.

HA--d htt MIMIC line. The d htt genome-tagging line with an in-frame "TagRFP-3xHA" tag was generated as described previously [34]. We used the parental MIMIC insert line MI01636, which contains a MIMIC insert in an intron region in the d htt gene. The proper expression of the full-length tagged d htt protein was confirmed by western blot with anti-HA and anti-d htt antibodies.

Plasmids. The following plasmids were from the Addgene: HAp62, MycLC3, FLAG--Atg13, HA--ULK1, FLAG--ULK1, Myc--ULK1-K46I, Myc--mTOR, Myc--mTOR-KD, Myc--Raptor, HA--Ub-K63, HA--Ub-K48, FLAG--Atg101, Myc--Rheb, AMPK, AMPK--9E10, AMPK--12CA5, AMPK--Roche, AMPK--Santa Cruz, AMPK--Cell Signaling; mouse anti-HA (12CA5, Roche); mouse anti-c-Myc (9E10, Santa Cruz); rabbit anti-p62 (2972, Cell Signaling); rabbit anti-Htt (1:1,000 for immunoblotting and 1:200 for western blotting with anti-HA and anti-Htt antibodies).

Fly tissues. Including larva eye imaginal discs and adult brains, were dissected and fixed in PBS as described previously [35]. Z--stack scanning of the dorsal thorax for each fly was recorded under a x10 objective using a Zeiss Axioimager Z1 microscope (usually 10–20 layers scanned) and reconstructed into a 3D projection using C Zen software.

Climbing assay. Climbing assays were performed as described previously [36]. Briefly, 30 to 50 flies were placed into the first chamber in the assay slider, tapped to the bottom, and then given 30 s to climb a 10 cm distance. Flies that successfully climbed 10 cm or beyond within 30 s were then shifted to a new chamber, and both sets of flies were given another opportunity to climb the 10 cm distance. This procedure was repeated for a total of at least five times. After five trials or more, the number of flies in each chamber was counted. The climbing index was calculated by measuring the partition coefficient (Cf), as described previously [36].

Viability assay. Viability assays were performed as described previously [36]. Briefly, 30 newly hatched female flies of a specific genotype were placed into individual vials with fresh food food. Flies were transferred into a new vial with fresh food every 2–3 days to prevent them from sticking to old food or becoming dehydrated.

Larva starvation assay. L2 or early L3 stage larva (n = 10) were collected and starved in 20% sucrose for 4 h. Fat tissues were dissected in PBS and fixed in 4% paraformaldehyde as described previously [34]. mCherry--atg8-positive puncta were recorded under the confocal microscope followed by quantitative analysis. Starvation resistance assay for adult flies. Newly enclosed or 5-day-old female flies raised over standard culture medium were transferred to fly vials (30 flies per vial) containing 0.3% agarose in PBS for the starvation test. Dead flies were counted every 4 h and the survival rate was calculated accordingly.

Proteasome peptide assay. Whole-fly extracts (30 flies per group) were prepared on ice by homogenization in 0.01 M Tris--EDTA, pH 7.5 buffer, followed by centrifugation at 20,817 g for 15 min. Total protein concentrations were determined by Bradford assay. Peptide activities were measured by recording the hydrolysis of the fluorogenic peptides Suc--LLVY--AMC (Anaspec, 63892), Boc--Leu--Arg--Arg--AMC (Enzo, BML-BW8513-0005), and Z-Leu-Leu-Glu--AMC (Enzo, BML-BW8515-0005), and Z-Leu-Leu-GLu–JNA (Enzo, BML-ZW8520-0005) as previously described [37]. Fluorescent dyes and immunofluorescent staining. Fly tissues, including larva eye imaginal discs and adult brains, were dissected and fixed in PBS as described.
previously\(^6\). Frozen sections or fixed tissues or cells were blocked in 10% normal goat serum at room temperature for 1 h followed by incubation in primary antibodies at 4 °C overnight, then stained with Alexa-594- or Alexa-488-conjugated secondary antibodies (Jackson ImmunoResearch). Washed samples were mounted in 80% glycerol and 20% DABCO for 30 s before analysis. Images were captured using a Carl Zeiss Axiovert 200 fluorescence microscope equipped with a ×63 Objective and ApoTome.2. Co-localization was determined using the JACOp Plug-In on ImageJ (NIH).

Transmission electron microscopy, immunogold labelling and morphometric analysis. Cells were cultured in monolayers, trypsinized, washed and subsequently fixed with 2.5% glutaraldehyde, and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.4 for 3 h at 4 °C. Cells were post-fixed with 1% osmium tetroxide, dehydrated with graded series of ethanol, and then infiltrated and embedded using Epon 812 (Electron Microscopy Sciences). Ultrathin sections (70–80 nm) were cut using a Leica Ultracut and stained using uranyl acetate and contrasted with lead citrate. Samples were viewed under a Jeol JEM-1200EX transmission electron microscope (Jeoel) at 80 kV. Morphometric analysis was done using ImageJ (NIH). Briefly, cytosolic area and individual autophagic vesicle areas were calculated after tracing the membrane profiles using the measure function of the ImageJ software. Classification of autophagic vacuoles according to their luminal content was done by double-blinded independent observers using one single-category allocation for each vesicle. Immunogold labelling was performed on cells fixed with 0.1% glutaraldehyde, 4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 for 3 h at 4 °C. Cells were dehydrated with graded series of ethanol, and then infiltrated and embedded using LR White (Polysciences). Ultrathin sections were cut as described above. Grids were aldehyde-inactivated with glycine–PBS, blocked with anti-goat AURION blocking solution, incubated with primary and the corresponding 10-nm-gold-conjugated secondary antibodies.

Western blotting. Standard 10 to 14% SDS–PAGE gels were used for separation of most proteins except for Huntingtin, which was better analysed by NuPAGE Tris-Acetate gels from Invitrogen specially formulated for detection of proteins with a large molecular weight. The boiled samples were separated on SDS–PAGE and transferred to nitrocellulose membranes from Millipore. After blocking with 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 for 1 h, membranes were incubated with primary antibodies. Secondary antibodies conjugated with Alexa-800 or Alexa-680 (Invitrogen) or HRP (KPL) were used and the signals were detected by the Odyssey Infrared Imaging System or LAS 300 Imaging system (Fujifilm) and quantified by Odyssey Application Software 3.0 or by densitometry of the digital images using ImageJ software (NIH).

Ubiquitylated proteins analysis. Ubiquitylated proteins analysis was conducted as previously described with a minor modification\(^6\). Briefly, about 20–30 adult fly heads of indicated ages were collected and the whole protein lysates were extracted using ubiquitylated protein extraction buffer (2% SDS with 6 M urea) with brief sonication. The profile of ubiquitylated proteins from the extract was revealed by western blot using mouse monoclonal anti-ubiquitin antibody (Clone FK2, Millipore) in 12% SDS–PAGE containing 6 M urea.

GST–BHMT assay. The GST–BHMT assay was performed as described previously\(^6\). Briefly, pPK5–GST–BHMT was co-transfected with different siRNAs into cells as indicated in the study, which were then treated with different assay conditions such as nutrition starvation using EBSS or proteasome inhibition using MG132. The accumulation of GST–BHMT–FRAG was detected by using mouse anti-GST antibody (B14, Santa Cruz) and normalized against the internal control GFP protein, which was expressed after the internal ribosome site (IRES) in the same pPK5–GST–BHMT construct.

Autophagic measurements. All autophagy assays were carried out as previously described\(^6\). For the LC3 lipidation assay, cells were collected in 2% Triton X-100/PBS buffer containing protease inhibitors for maximum LC3-II extraction according to the previous studies\(^6\). LC3-I and LC3-II were detected by rabbit anti-LC3 antibody (MBL international) and mouse anti-actin antibody (Chemicon), respectively. The level of LC3 lipidation was quantified as the ratio of the measured LC3-II to actin levels. LC3 flux was quantified as the relative ratio of LC3-II/actin values between samples with or without the presence of lysosome inhibitor BafA1 or a mixture of ammonium chloride (20 mM; American Bioanalytical) and leupeptin (100 mM; Fisher Bioagents). LC3-II synthesis (autophagosome biogenesis) was calculated as the differences in LC3-II levels at two different times on inhibition of lysosomal proteolysis as described previously\(^6\).

For the LC3 puncta formation assay, cells were fixed by paraformaldehyde (10 min at room temperature, RT), permeabilized by 50 μg ml\(^{-1}\) digitonin (10 min, RT), quenched by 50 mM NH\(_4\)Cl (freshly made, 5 min, RT) and followed by standard antibody staining. The primary and secondary antibodies used were rabbit anti-LC3 antibody (MBI international) and Alexa-488 anti-rabbit IgG (Jackson ImmunoResearch Laboratories). For each assay condition, the puncta profile data were calculated by averaging the total number of LC3-positive puncta per cell from about 50–100 representative cells.

For McCherry–GFP–LC3 conversion, NIH3T3 fibroblasts, MEFs, N2a and striatal cells were transduced with a lentivirus carrying the tandem construct and cells were analysed at least one week after transduction to assure stable expression. Cells were plated in glass-bottom 96-well plates and fluorescence after the indicated treatment was read in both channels using a high-content microscope (Operetta, Perkin Elmer). Images of 9 different fields per well were captured, which rendered an average of 2,000–3,000 cells counted. Nuclei, cell perimeter and puncta were identified using the manufacturer’s software. Puncta positive for both fluorophores correspond to autophagosomes whereas those only positive for the red fluorophore correspond to autolysosomes. Autophagic flux was determined as the conversion of autophagosomes to autolysosomes (red-only puncta).

For mCherryGFPLC3 conversion, NIH3T3 fibroblasts, MEFs, N2a and striatal cells were treated with a lentivirus carrying the tandem construct and cells were analysed at least one week after transduction to assure stable expression. Cells were plated in glass-bottom 96-well plates and fluorescence after the indicated treatment was read in both channels using a high-content microscope (Operetta, Perkin Elmer). Images of 9 different fields per well were captured, which rendered an average of 2,000–3,000 cells counted. Nuclei, cell perimeter and puncta were identified using the manufacturer’s software. Puncta positive for both fluorophores correspond to autophagosomes whereas those only positive for the red fluorophore correspond to autolysosomes. Autophagic flux was determined as the conversion of autophagosomes to autolysosomes (red-only puncta).

For LysoTracker Red treatment, NIH3T3 fibroblasts, MEFs, N2a and striatal cells were treated with a lentivirus carrying the tandem construct and cells were analysed at least one week after transduction to assure stable expression. Cells were plated in glass-bottom 96-well plates and fluorescence after the indicated treatment was read in both channels using a high-content microscope (Operetta, Perkin Elmer). Images of 9 different fields per well were captured, which rendered an average of 2,000–3,000 cells counted. Nuclei, cell perimeter and puncta were identified using the manufacturer’s software. Puncta positive for both fluorophores correspond to autophagosomes whereas those only positive for the red fluorophore correspond to autolysosomes. Autophagic flux was determined as the conversion of autophagosomes to autolysosomes (red-only puncta).

RNA interference. The duplex RNA oligonucleotides for siRNA (Sigma) were used at 1–10 nM for knockdown of target genes in HEK293T or HeLa cells, as indicated. The sequences for the sense oligonucleotides were: Htt-1 sense siRNA (5′-acacuacauccacauccagg-3′; ref. 80) and Htt-2 sense siRNA (5′-ccaggauauuuuuagc-3′) (validated sequence from Qiagen); beclin1-1 sense siRNA (5′-acccacucaacuucagggaa-3′); and beclin1-2 sense siRNA (5′-caggauaucacuuccagggaa-3′); Ctrl siRNA (negative control from Qiagen). Lentivirus constructs carrying shRNA against Huntingtin were generated as previously described\(^6\) and delivered to cultured cells on packaging into replication-deficient lentiviral particles to generate lentiviral vector with either a non-HA, anti-Myc, anti-FLAG, anti-Htt, anti-p62 and anti-ULK1 antibodies and Protein A/G Plus-agarose beads (sc-2003, Santa Cruz) as indicated. Immunoprecipitates or whole-cell extracts (WCEs) were analysed by standard western blotting.
the proteins are included in the manuscript. We did not find differences between the two Ht shRNAs used in this study and consequently, where indicated, data from both clones were pooled.

**In vitro ULK1 kinase assay.** The in vitro ULK1 kinase assay was performed as previously described. Briefly, FLAG-ULK1 or FLAG–ULK1-K46I was transfected into HEK293T cells. Thirty-six to forty-eight hours after transfection, cells were lysed in TLB. Supernatants were incubated with M2 beads (Sigma) and washed three times by TLB followed by a wash using ULK1 kinase buffer (10 mM cold ATP, 25 mM HEPES, pH 7.4) before addition of γ-p-ATP to 0.5 μCi and myelin-binding protein (MBP) to 0.3 mg ml⁻¹ for 20 min of kinase reaction. To ensure reproducibility, during the in vitro kinase assay, the ULK1 protein applied to each sample was enriched through immunoprecipitation, and the final amount of ULK1 immunoprecipitated was evaluated by immunoblot, which verified only minor negligible variations among different samples. To stop the reaction, 4× SDS sample buffer was used, and this was followed by SDS–PAGE and autoradiographic exposure using X-ray film (Kodak).

**mTOR kinase assay.** Both, the in vivo and in vitro mTOR kinase assays were performed as previously described. Briefly, for the in vivo mTOR kinase assay, HEK293T cells in 60 mm dishes transfected with different plasmids or siRNAs as indicated were lysed in TLB. After denaturing in SDS sample buffer, samples were subjected to western blotting analysis using anti-p-S6K (Thr389) and anti-S6K antibodies for detection of the phosphorylation and expression levels of the endogenous S6K protein, respectively.

**Lipid metabolism assays.** Rates of fatty acid β-oxidation were determined by metabolic radioactive labelling as described earlier and by respiratory measurements. Briefly, cells pretreated with OL were incubated with [14C]oleate–BSA (0.8 μCi, 4 h) and the rate of carbon dioxide production resulting from the oxidation of [14C]oleate was measured on trapping the released [14C]carbon dioxide at 37 °C for 1 h onto filter paper pre-soaked in 100 mM sodium hydroxide. The rate of β-oxidation was calculated as the amount of trapped [14C]carbon dioxide in relative units produced per milligram of protein per hour. Equal numbers of cells were plated in XF96 plates (Seahorse Bioscience) in low-glucose DMEM (GIBCO) supplemented with serum. Cells were treated with OL for 16 h and cell respiration was assayed as time-resolved measurements of the oxygen consumption rate (OCR) in a respirometer (Seahorse Bioscience). Fatty acid β-oxidation was calculated as specified by the manufacturer by injecting etomoxir (50 μM) into the buffered assay medium.

**Inducible Tau-expression line.** The Clontech Lentri-X Tet-OFF advanced inducible expression system was used to generate the inducible Tau line in HeLa cells. Basically, HeLa cells were infected by mixed lentivirus containing pLX-Tet-off Advanced and pLX-Tight-Puro-TauΔC-GFP. Stable clones were selected against 1 μg ml⁻¹ puromycin and 200 μg ml⁻¹ G418 in the presence of 100 ng ml⁻¹ Dox to repress transgene expression. TauΔC–GFP expression can be inducibly expressed by removal of Dox.

**Statistics analysis and data acquisition.** The statistical significance of the difference between experimental groups was determined by two-tailed unpaired Student's t-test, or one-way analysis of variance followed by Bonferroni's post hoc test. Where multiple comparisons were performed, we used normalization to control values. Differences were considered significant for P < 0.05 (noted in the figures as *). Data are presented as mean ± s.e.m from a minimum of three independent experiments. The exact sample size (n) is indicated in each figure and it corresponds to individual experiments unless otherwise stated. All of the experiments were done at least 3 times and in duplicate or triplicate to account for technical variability. For the studies in Drosophila, sample group allocation was based on genotype and the genotypes were blinded to the observer except for those cases in which tissues from different animals have to be pooled. For the morphometric analysis, quantification of annotated micrographs was independently reviewed by two observers and the average of their scoring was used for each micrograph. For immunofluorescence and direct fluorescence quantifications of co-localization, number of puncta per cell and ratio of red to green fluorophore were performed blinded. For the studies of analysis of changes in different steps of the autophagic process an estimate of variation was performed based on previous studies from our groups and the variance was found similar to the one in the groups being statistically compared in this study.
**Supplementary Figure 1** Ectopic Tau expression reveals genetic interaction between dhtt and autophagy pathway. (a) Tau expression induced the collapsed thorax phenotype in homozygous dhttko-/- mutants (arrow in a3) that can be rescued by the presence of a dhtRescue transgene (a4). Bright field images of adult thorax, dorsal view (pooled from n=3 independent experiments). (b,c) Tau expression induced more severe mobility (b) and viability phenotypes (c) in dhttko mutants that can be rescued by the presence of a dhtRescue transgene (pooled from n=3 independent experiments). (d,e) dhtt genetically interacts with multiple autophagy pathway components. Bright field images of adult thorax, dorsal view. Tau expression induced the collapsed thorax phenotype both in homozygous dhttko-/- (arrow in a3) and in atg8ad4-/- (arrow in d7) mutant flies. Similar phenotype was also observed in Tau-expressing flies carrying double heterozygous mutations for both dhttko and one of the following mutant alleles: atg8ad4 (d5), ref(2)Pc003993 (e3) or atg1 ∆3D (e5), but not in all other controls. The penetrance of the phenotype was quantified as bar graphs to the right (d) or below (e). Genotypes are as indicated (pooled from n=3 independent experiments). All values are mean+s.e.m. and differences are significant for *P<0.05 using analysis of variance + Bonferroni test. Scale bars: 100 µm
**Supplementary Figure 2** *dhttk* does not affect proteasome activity and starvation response in *Drosophila*. (a) Increased accumulation of total ubiquitinated proteins in aged (5-week-old) *dhttk*-/* mutants compared to aged-matched controls (wildtype (WT) and *dhttk*-/* carrying a wildtype *dhttk* rescue transgene (*dhttk*recovery). Young flies (1-week-old) showed similar low levels of total ubiquitinated proteins in all the three genotypes. Bar graph below shows the quantification of the levels of total ubiquitinated proteins detected with anti-ubiquitin antibody by western blotting (n=3 independent experiments). (b) and (c) Abnormal eye phenotypes (b) and increase ubiquitination (c) associated with proteasome dysfunction. (b) Bright field images of adult fly eyes. Note the rough eye phenotype due to proteasome inactivation by DTS5 or DTS7, two temperature-sensitive, dominant-negative alleles of proteasome subunits at the non-permissive 28°C, as compared to the normal eye morphology in flies of the same genotypes raised at permissive temperature of 22°C (compare a2 and a3 with a5 and a6). GMR-Gal4 driver was used to direct eye-specific expression of UAS-DTS5 and DTS7. LacZ was used as negative control (a1 and a4). Scale bar: 50 μm. Anterior up and ventral to the right in all panels. n>15 fly samples from 3 independent experiments. (c) Total proteins from flies of the indicated genotypes were extracted by lysis buffer containing 1% Triton X-100 and separated by 10% SDS-PAGE gel. The level of ubiquitination was revealed by anti-Ub antibody (FK2). Actin served as loading control. Note the significantly increased levels of ubiquitinated proteins in flies expressing the dominant-negative DTS7 mutant driven by arm-Gal4 under restrictive temperature of 28°C but not at 22°C or in controls (n=3 independent experiments). (d) Normal thorax morphology (top panels) and internal muscle structures (bottom panels) in flies expressing Tau together with the dominant-negative DTS5 or DTS7 alleles of proteasome subunits at either permissive 22°C or non-permissive 28°C temperatures, as indicated. Top panels: bright field images of external thorax morphology, bottom panels: cross section views of underlying muscle structures, as revealed by confocal imaging of F-Actin patterning from phalloidin staining (bottom). Note the similar smooth thorax surface and intact internal muscle structures in all panels. Simultaneous expression of Tau and DTS5 or DTS7 were driven by A307-Gal4 line, as indicated. Scale bar: 100 μm. n>10 fly bodies from 3 independent experiments. (e) Normal UPS activity in *dhttk*-/* mutants. No obvious accumulation of UPS reporter GFP-CL1 signal in *dhttk*-/* mutants in either the eye imaginal discs of third instar larva (c2 and c4) or 3-day-old adult brains (c6 and c8) at either 22°C or 28°C. In contrast, in the positive controls that expressed a temperature-sensitive dominant-negative UPS mutant (DTS7) at a non-permissive temperature of 28°C (c3 and c7), there is a strong accumulation of the UPS reporter GFP-CL1. Scale bar: 100 μm. n>8 fly samples from 3 independent experiments. (f) Normal catalytic activities of proteasome subunits in *dhttk*-/* mutants. Caspase-like, trypsin-like and chymotrypsin-like peptidase activities were measured using whole-animal extracts from young (5-day-old) or old (40-day-old) wild-type and *dhttk*-/* flies, and their relative activities are presented with results from young wildtype flies as standard value of 1. n.s.: no statistical significance. n=18 fly extracts for each genotype, pooled from 3 independent experiments. (g, h) *dht* is not required for the starvation-induced autophagy. Autophagosomes and autolysosome in fat bodies of 80-hour larvae were labeled with mCherry-Atg8a (red). Starvation induced similar strong increase of red puncta (autophagosomes and autolysosome) in both the wildtype and *dhttk*-/* mutants, as quantified in the bar graphs to the right. UAS-mCherry-Atg8a reporter was driven by the fat body-specific Gq-Gal4 driver. Tissues were double-labeled with DAPI (blue) for cell nuclei. (h) Bar graph shows the quantification of the average number of mCherry-Atg8a-positive punctae. n=60 cells for each genotype, pooled from 3 independent experiments. (i) Starvation-resistance assay. Newly eclosed or 5-day-old adult flies were subjected to starvation. *dhttk*-/* mutants showed similar survival curve as wildtype control (CS, Canton S), n=155 flies (newly eclosed CS), n=160 (newly eclosed *dhttk*/*), n=203 (5 days CS), n=180 (5 days *dhttk*/*), pooled from 3 independent experiments. All values are mean+s.e.m. and differences are significant for *P<0.05 using student’s t-test or analysis of variance + Bonferroni test.
**Supplementary Figure 3** Human Htt rescues *dhtt* mutant-associated phenotypes. (a) Schematics of the C-terminus truncated Tau (Tau-ΔC), missing last 49 amino acids) used in the study, as compared to the full-length (FL) Tau. Below: western blotting analysis to confirm the expression of Tau deletion used in the figure and were pooled from n=4 independent experiments.

(b) Confirmation of the proper expression of ubiquitously expressed Tau and hHtt as well as the human full-length Huntingtin (hHtt) and GFP proteins from the corresponding transgenes by Western blotting analyses. The ubiquitous arm-Gal4 driver was used to drive the ectopic Tau expression, protein expression was examined by anti-GFP antibody, with Actin served as loading control.

(c) Ectopic expression of full-length human Huntingtin (hHtt) significantly rescued several *dhtt* associated phenotypes, including (c) the reduced mobility and (d) shortened lifespan of mutant adults. N values are indicated in the figure and were pooled from 4 independent experiments. (e and f) Ectopic expression of hHtt significantly rescued Tau-induced phenotypes in *dhtt* mutant background, including the exacerbated (e) mobility decline and (f) reduced lifespan. A307-Gal4 driver was used to drive the Tau-ΔC-GFP and hHtt expression. N values are indicated in the figure and were pooled from n=4 independent experiments.

(g-i) hHtt partially suppressed the increased accumulations of (g-h) total ubiquitinated proteins and (f) Tau-ΔC in *dhtt* mutant flies. Ubiquitous arm-Gal4 driver was used to drive the hHtt expression; Tau-ΔC level was examined by anti-GFP antibody, with Actin serving as loading control. n=3 independent experiments. All values are mean±s.e.m. and differences are significant for *P<0.05 using analysis of variance + Bonferroni test.
Supplementary Figure 4 Htt knockdown does not affect proteasomal activity or lysosomal function. (a-c) Htt knockdown does not affect the proteasome activity in mammalian cells. Proteasome activity in HEK293T cells was evaluated by examining the level of (a) a UPS reporter CL1-GFP and (b) ubiquitinated β-catenin, a bona fide substrate of proteasome. Htt knockdown by two independent siRNA did not cause an abnormal accumulation of (a) GFP-CL1 (lanes 3 and 4) or (b) ubiquitinated β-catenin, resembling that from samples treated with the control siRNA (Ctrl, lane 2) or autophagy inhibitor BafA1 (lane 6). In contrast, treatment with proteasome inhibitor MG132 induced a higher level of both (a) GFP-CL1 (lanes 5) and (b) ubiquitinated β-catenin. BafA1 was applied at 100 nM for two hours and MG132 was applied at 10 μM for 6 hours before the assay. n=3 independent experiments. (c) Normal catalytic activities of proteasome subunits in Htt knock down NIH3T3 fibroblasts. Caspase-like, trypsin-like and chymotrypsin-like peptidase activities were measured using whole cell extracts from HEK293T cells treated with si-Ctrl, si-Htt or MG132. Their relative activities are presented with results from si-Ctrl cells as the standard of 1. n.s.: no statistical significance. n=3 independent experiments. (d-h) Htt knockdown does not affect lysosomal function. (d) Staining of acid compartments with LysoTracker does not reveal problems of acidification in lysosomes although in agreement with the electromicroscopy studies shows some level of expansion of these acid compartments. (e-f) Internalization of fluorescent transferrin is comparable in both cell types supporting the absence of major alterations in the endocytic system. (e) Representative images of transferrin loaded cells. Inserts: higher magnification images of the transferring channel and (f) quantification of the number of transferrin positive puncta per cell. n = 3 independent experiments where 80 cells were examined in total from 3 independent fields in each condition. (g,h) Levels of LAMPs are not reduced in Htt(-) cells, in fact a slight increase in levels of LAMP1 as measured by immunoblot (g) and of LAMP-2 positive puncta (h) was noticeable in Htt(-) cells either in the presence or absence of serum. Scale bar: 10 μm. (i-k) Time-course analyses of the levels of Tau-ΔC-GFP protein expressed from stably transfected Dox-inducible HeLa cell line. Degradation of Tau-ΔC-GFP was significantly delayed by (i) autophagy inhibitor 3-MA or (j) by siRNA-mediated knockdown of ATG7, whereas (k) proteasome inhibitor MG132 showed little effect. Control cells were treated with only water (i), scramble siRNA (j) or DMSO (k). Quantification of multiple repeat experiments is shown at the bottom n=9 dishes for each condition pooled from 3 independent experiments. The difference was most evident around 48 hours after induction of Tau-ΔC-GFP expression, which was detected by anti-GFP antibody. Actin served as loading control. In i and j, additional panels at the bottom show reduced LC3 lipidation upon addition of 3-MA or Atg7 siRNA supporting the efficacy of these treatments in blocking autophagy (note that due to the low basal steady-state levels of LC3-II in these cells, Baf1 was added to detect changes of these treatments in blocking autophagy). All values are mean+s.e.m. and differences are significant for *P<0.05 using analysis of variance + Bonferroni test (c) or student’s t-test (i,j,k,l).
Supplementary Figure 5 Effect of Htt depletion on autophagic flux in mammalian cells. (a, b) Htt does not affect the basal level of autophagic activity in dividing mammalian HEK293T cells. (a) LC3 lipidation assay. Compared to controls, Htt knockdown did not have obvious effect on the level of lipidated LC3 (LC3-II) either in the absence or the presence of lysosomal inhibitor BafA1, which blocks autophagy flux and increases cellular LC3-II levels. (b) GST-BHMT assay. Htt knockdown in the same cells did not show obvious effect on the level of the cleaved GST-BHMT-FRAG product. MG132-treated samples were included as positive controls (lanes 5 and 10 in (a) and lane 5 in (b)), which showed robust induction of LC3-II and GST-BHMT fragmentation. Actin served as loading control. n=3 independent experiments.

c) Restored autophagosome formation by GFP-Htt overexpression in Htt-knockdown HeLa cells. Immunofluorescent staining for endogenous LC3 (red) in HeLa wildtype (Ctrl), or si-Htt interfered cells (Htt-) untreated or co-transfected with GFP-Htt cDNA (GFP-Htt). Cells were cultured in the presence or absence of MG132 for 6 hours and/or BafA1 for 2 hours before harvesting. Overexpression of GFP-Htt, confirmed by anti-GFP antibody staining (green), rescued the reduced number of LC3-positive puncta in MG132-treated Htt(-) cells, as shown by quantification at the bottom. n = 3 independent experiments where a total of 120 cells were analyzed per condition. Scale Bar: 5 μm

d) Restoration of Htt in MEFs from Htt knockout (Htt-KO) mice rescues defective autophagosome biogenesis during induction of selective autophagy. Htt-KO MEFs were transiently transfected with a plasmid carrying GFP-Htt and subjected to the indicated treatments. Images show staining for LC3 with a Cy5-conjugated secondary antibody. Representative regions containing a transfected and untransfected cell are shown as merge channels (left) and red channel only (bottom). Nuclei are highlighted with DAPI. Scale Bar: 5 μm. Bottom shows quantification of the average number of LC3 puncta per cell in both cell populations. n = 3 independent experiments where a total of 120 cells were analyzed per condition. Htt affects MG132-induced autophagosome biogenesis but not its fusion with lysosomes. HeLa cells were double labeled for LC3 (green) and lysosomes (LAMP1 (red). BafA1 treatment led to a significant lysosomal accumulation of LC3. Knockdown of Htt expression by two independent siRNA both significantly suppressed the MG132-induced formation of LC3-positive puncta, but did not affect the accumulation of remaining LC3 in the lysosomes. The levels of co-localization between LC3 and LAMP1 are quantified in bar graphs (bottom). Scale Bar: 2 μm. n = 3 independent experiments where a total of 45 (in none), 90 (in MG132) and 100 (in Serum-) cells were analyzed per condition. (f) Htt does not affect starvation-induced autophagy in dividing mammalian HEK293T cells. Compared to controls, Htt knockdown did not have obvious effect on the level of lipidated LC3 (LC3-II) either in the absence or the presence of lysosomal inhibitor BafA1. HeLa cells cultured in nutrient rich medium (RM) or EBSS medium were used for the studies. Untreated (RM) and control siRNA (si-Ctrl)-treated samples were included as negative controls. n=3 independent experiments.

g-k) Depletion of Htt in different types of mammalian cells reduces selective autophagic flux but not starvation-induced autophagic flux. Control and Htt knock-down (Htt(-)) NIH3T3 (g), N2a (i) or mouse striatal derived (j,k) cells and MEFs from wild-type or Htt knock-out mice (Htt KO) (h) were transduced with a vector encoding mCherry-GFP-LC3 and subjected to the indicated stimuli to activate nutrient-induced autophagy (serum- and EBSS) or selective autophagy in response to proteostasis (Lactacystin, MG132), lipotoxicity (oleic) or mitochondria depolarization (FCCP) as labeled. Cells were imaged using high content microscopy and autophagic flux was determined as conversion of autophagosomes (yellow puncta) into autolysosomes (red only puncta). n = 3 independent experiments where a total of 2250 (g-i) and 3000 (j) cells were analyzed per condition. Representative images of mouse striatal-derived cells upon the indicated treatments. Merged channels are shown. Nuclei are highlighted with DAPI. Scale bar: 5 μm. All values are mean+s.e.m. and differences are significant for *P<0.05 using analysis of variance + Bonferroni test (e) or student’s t-test (c,d,g-j).
Supplementary Figure 6 Structural-functional analysis of Htt interaction with p62 and ULK1 proteins. (a-d) Htt physically interacts with p62 and ULK1 in HEK293T cells. Reciprocal co-IP experiments between (a and b) HA- or FLAG-tagged Htt with FLAG- or HA-tagged p62, or (c and d) between HA- or Myc-tagged Htt with FLAG- or HA-tagged ULK1, as indicated. Htt was detected in the same immunoprecipitates both with p62 and with ULK1 in co-IP assays from either direction (lanes 2). Lanes 4 are positive controls for Myc-tagged mHAP1 (a and c), a known Htt-associated protein, Myc-tagged LC3 (b), a known p62 interactor, or FLAG-tagged Atg13 (d), a known ULK1 binding partner. n=3 independent experiments. (e) Schematics of putative functional and structural motifs in human Htt protein: N17 (grey color): the first 17 amino acids of Htt protein, which is right in front of the polyglutamine tract (polyQ, blue color); PolyP (yellow): the proline-rich region adjacent to polyQ tract; NES: nuclear export signal. The predicted 40 HEAT repeat in the Htt protein was presented as red cylinders and numbered accordingly. (f) Schematic illustration of the five most conserved regions in human Htt protein (labeled alphabetically from “a” to “e”) that bear the highest sequence similarity with its Drosophila Htt counterpart. (g) Schematics of the nine Htt deletions used in the study (top), designated as NT (N-terminal), MD (middle), CT (C-terminal), and D2 to D7 regions, as illustrated. The deletions were designed based on the distribution of the predicted structural motifs and conserved sequences as illustrated in (e) and (f).
**Supplementary Figure 7** Htt facilitates the cargo recognition efficiency by p62 protein. (a) Time-course analysis of p62 distribution in Triton X-100 soluble (top) and insoluble fractions (middle), as well as total p62 protein (bottom) in HEK293T cells after MG132 treatment, as indicated. Actin served as loading control. Note the gradual decrease of soluble p62 with concomitant increase of insoluble p62, as well as the marked increase of total p62 at hour 12. n=3 independent experiments. (b and c) MG132-induced turnover of soluble p62 is autophagy-dependent. (b) Beclin 1 knockdown in HEK293T cells suppressed MG132-induced depletion of Triton X-100-soluble p62 (compare lanes 3 with 2). 5 nM siRNA against beclin 1 was effective in reducing the expression of Beclin 1 protein (middle panels). (c) Bar graph representation of the soluble p62 levels, which were quantified and normalized against loading control Actin, as indicated. Scale bars indicate s.e.m. n=9 dishes for each condition pooled from 3 independent experiments *p<0.05 using analysis of variance + Bonferroni test. (d) Htt does not affect p62 level under basal and starvation conditions in HEK293T cells. Under both conditions, si-Htt treated samples showed similar levels of p62 protein as in controls of mock- or control siRNA-treated samples. Note the overall reduced level of p62 protein under starvation condition (EBSS incubation), n=3 independent experiments. (e) Htt does not affect p62 self-polymerization. *in vitro* co-IP assay to assess the interaction between HA- and Myc-tagged p62 in transfected HEK293T cells. Similar levels of Myc-p62 were pulled down by HA-p62 between the siHtt-treated (lanes 3 and 4) and control siRNA-treated (lane 2) samples. n=3 independent experiments. (f-i) Htt specifically affects the binding affinity between endogenous p62 protein and substrates with Ub-K63, but not Ub-K48, modifications. Co-IP experiments against endogenous ubiquitin using (f) anti-Ub-K63 or (g) anti-Ub-K48 from HEK293T cells, or using anti-HA antibody from HEK293T cells transfected with HA-K63 (HA-Ub-K63) or (i) Ub-K48 (HA-Ub-K48). In both cells, Htt knockdown significantly reduced the amount of endogenous p62 that co-immunoprecipitated with Ub-K63 modified proteins, but showed no obvious effect on that co-immunoprecipitated with Ub-K48 modified proteins (compare lanes 2 and 3 with lane 1 in both (f) and in (g), and in both (h) and in (i)). n=3 independent experiments.
Supplementary Figure 8 Htt forms distinctive complex with ULK1. (a and b) Htt does not affect the binding and phosphorylation of ULK1 by AMPK. (a) Co-IP experiment using anti-FLAG antibody to examine the physical interactions between ULK1 and AMPK, as indicated. HEK293T cells were transfected with FLAG-tagged ULK1 together with HA-tagged AMPKα, AMPKβ, AMPKγ1 subunits. Htt knockdown showed no effect on the levels of AMPK subunits co-immunoprecipitated with ULK1. WCE: whole cell extract. (b) Htt knockdown in HEK293T cells did not affect the phenformin-stimulated phosphorylation of ULK1 at the targeted Ser555 site (ULK1-S555) by AMPK (compare lanes 3 and 4 with lane 2). Samples were treated in 1 μM phenformin for 2 hours before the assay. A constitutive active AMPK (AMPK-CA) was included as the positive control for the kinase assay (lane 5). n=3 independent experiments. (c) Htt knockdown does not affect the stability of the ULK1-FIP200-Atg13-Atg101 complex. Co-IP experiment in HEK293T cells to assess the effect of Htt knockdown on the physical associations among the ULK1 complex components. HA-tagged ULK1 was co-transfected with other ULK1 complex components FIP200, Atg13 and Atg101, followed by co-IP using anti-HA antibody, as indicated. Htt knockdown by two independent siRNA showed no obvious effect on the binding affinity among these ULK1 complex components, resembling that observed from the control siRNA treated sample (compare lanes 3 and 4 with lane 2). n=3 independent experiments. (d-f) Htt does not affect mTOR kinase activity. (d and e) in vivo mTOR kinase assay. Neither Htt knock-down nor (e) Htt overexpression in HEK293T cells showed any obvious effect on the mTOR kinase activity in vivo. (e) Htt overexpression also did not affect basal mTOR kinase activity (compare lane 3 and 4 with lane 1) or Rheb-induced mTOR activation (compare lane 5 with lane 2). The phosphorylation level of S6K at Thr389 (p-S6K (Thr389)) was used as the readout of in vivo mTOR kinase activity. Upstream mTOR activator Rheb was included as the positive control for the kinase assay. (f) in vitro mTOR kinase assay. Htt knockdown did not affect mTOR kinase activity in vitro (compare lanes 3 and 4 with lane 2). 4E-BP1 was used as the substrate and its phosphorylation level at position Ser65 (p-4E-BP1 (S65)) was used as the readout of in vitro mTOR kinase activity. A dominant-negative kinase dead (KD) mutant of mTOR (Myc-mTOR-KD) was included as negative control for the assay. n=3 independent experiments. (g-i) Endogenous co-IP experiments in HEK293T cells using (g) anti-ULK1, (h) anti-Htt or (i) anti-mTOR antibodies. (g) ULK1 co-immunoprecipitated with both endogenous Htt and mTOR (lane 4). (h) Htt co-immunoprecipitated with endogenous ULK1 but not mTOR (lane 4). (i) mTOR co-immunoprecipitated with endogenous ULK1 but not Htt (lane 4). Cells treated with siRNA against ULK1 (g), Htt (h) or mTOR (i) were included as negative controls. n=3 independent experiments. (j and k) Increased ULK1-Htt complex and concomitant loss of ULK1-mTOR complex after lipotoxic or mitotoxic challenges. HEK293T cells co-transfected with Myc-mTOR and Flag-ULK1 were incubated with (j) 200μM Oleic acid (OA) for 12 hours or (k) with 10μM FCCP for 1 hour before harvesting for co-IP experiments using anti-FLAG antibody. Under either basal or OA and FCCP challenges, FLAG-tagged ULK1 could immunoprecipitate both Myc-tagged mTOR and endogenous Htt. However, both (j) lipid and (k) mitochondrial stresses led to a significant higher levels of Htt and the simultaneous loss of mTOR (compare lanes 7 and 8 with lane 6 in (j) and (k)), while Htt knockdown largely restored the ULK1 association with mTOR (compare lanes 7 and 8 with lane 6 in (j) and (k)). n=3 independent experiments. (l, m) Elevated Htt protein levels in response to induction of selective autophagy. Western analyses of Htt protein levels in NIH 3T3 (l) and N2a neuroblastoma (m) cells that were mock treated (None), serum deprived (serum -), or exposed to lactacystin (Lacta), MG132, FCCP or oleic acid, as indicated. Graph in l shows quantification of changes in Htt levels in NIH3T3 cells (n= 7 different cellular extracts for condition from 5 independent experiments, error bars indicate s.e.m., and *p<0.05 compared to None using student’s t-test). Actin served as loading controls.
Supplementary Figure 9 Uncropped images of the original scans of immuno-blots presented in the main figures. Red boxes indicate the cropped portion of each immunoblot presented in the corresponding main figure. Ladder of molecular weight markers is show on the left of each panel. Color codes are as follows: Blue 130kDa, Green 95kDa, Purple 72 kDa, Red 55 kDa, Cyan 43 kDa, Orange 32 kDa, Grey 26 kDa, Pink 17kDa.
Supplementary Figure 9 continued
Supplementary Figure 9 continued