Ubiquilins regulate autophagic flux through mTOR signalling and lysosomal acidification

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Although the aetiology of amyotrophic lateral sclerosis (ALS) remains poorly understood, impaired proteostasis is a common feature of different forms of ALS. Mutations in genes encoding ubiquilins, UBQLN2 and UBQLN4, cause familial ALS. The role of ubiquilins in proteasomal degradation is well established, but their role in autophagy–lysosomal clearance is poorly defined. Here, we describe a crosstalk between endoplasmic reticulum stress, mTOR signalling and autophagic flux in Drosophila and mammalian cells lacking ubiquilins. We found that loss of ubiquilins leads to endoplasmic reticulum stress, impair mTORC1 activity, promotes autophagy and causes the demise of neurons. We show that ubiquilin mutants display defective autophagic flux due to reduced lysosome acidification. Ubiquilins are required to maintain proper levels of the V0a/V100 subunit of the vacuolar H+–ATPase and lysosomal pH. Feeding flies acidic nanoparticles alleviates defective autophagic flux in ubiquilin mutants. Hence, our studies reveal a conserved role for ubiquilins as regulators of autophagy by controlling vacuolar H+-ATPase activity and mTOR signalling.

Ubiquilin (UBQLN) proteins are highly conserved ubiquitin-binding proteins. Although flies have a single ubiquilin gene (ubqn), there are five human homologues: UBQLN1, UBQLN2, UBQLN3, UBQLN4 and UBQLN5. Mutations in UBQLN2 and UBQLN4 cause ALS with or without frontotemporal dementia (FTD)2–5.

Ubiquilins are characterized by a carboxy-terminal ubiquitin-associated (UBA) domain and an amino-terminal ubiquitin-like (UBL) domain that mediates interaction with the proteasome4. The middle region between the UBL and UBA domains contains a variable number of poorly characterized chaperone-binding motifs homologous to STI1. Ubiquilins are suggested to play a role in diverse biological processes such as cell signalling, cell cycle progression, endoplasmic reticulum (ER)-associated degradation, autophagosome maturation and starvation-induced autophagy3–6. Research in human cell lines suggests a role for ubiquilins in chaperoning mitochondrial membrane proteins and protein aggregate clearance via heat shock protein 70 (HSP70) and the proteasome3,6,9. Despite our understanding of the role of ubiquilins in the ubiquitin–proteasome system, their role in autophagy is ill-defined and controversial6,7.

Here, we identified Drosophila Ubqn as a regulator of ER quality control, the mammalian target of rapamycin (mTOR) signalling, autophagy and neuronal maintenance. We report a dual function of Ubqn that integrates the ubiquitin–proteasome system and lysosomal degradation. We found that loss of Ubqn impairs mTOR complex 1 (mTORC1) activity and leads to increased autophagy induction in both flies and mammalian cells. Despite the promotion of autophagic vesicle formation, loss of Ubqn causes impaired autophagic flux. Ubqn interacts with subunits of the lysosomal proton pump, the vacuolar H+-ATPase (v-ATPase), and regulates v-ATPase function. Loss of Ubqn causes lysosomal acidification and affects lysosomal degradation due to impaired v-ATPase activity. Re-acidification of lysosomes with acidic nanoparticles (aNPs) ameliorates the impaired autophagic flux in ubqn mutants. Our data reveal a previously undocumented function for ubiquilins in autophagy regulation by promoting v-ATPase activity and lysosomal acidification, which in turn may play a role in the demise of neurons.

Results

Ubqn is broadly expressed and required in the developing nervous system. To isolate genes required for neuronal maintenance in Drosophila, we performed an unbiased forward genetic screen6,11. We identified a single nonsense mutation (Q129X) in ubiquilin, ubqn1, a fly homologue of human ubiquilins12 (Fig. 1a; Supplementary Table 1). The Ubqn protein was ubiquitously expressed in developing larvae, but was most abundantly expressed in the larval and adult nervous systems, as gauged by a C-terminal green fluorescent protein (GFP)-tagged ubqn genomic construct13 (Fig. 1b; Supplementary Fig. 1a).

Both ubqn mRNA levels and Ubqn protein levels were significantly reduced in ubqn1 mutants (Fig. 1c,d). Ubqn loss caused developmental arrest as early as pre-pupae (Supplementary Table 2). The lethality was rescued with a 20-kb genomic rescue construct14 and with ubiquitous overexpression of ubqn complementary DNA (Supplementary Table 2; Supplementary Fig. 1b). A null allele of ubqn (ubqn1Δubqn), whereby the gene was deleted using the clustered regularly interspaced short palindromic repeats–CRISPR associated protein 9 (CRISPR–Cas9) technique15, did not complement ubqn1 lethality and showed pre-pupal lethality (Fig. 1a(i); Supplementary Fig. 1c and Supplementary Table 2). These data demonstrate that the ubqn1 is a severe loss-of-function or null allele of ubqn.

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Fig. 1 | Ubqn is broadly expressed and required in the developing and adult nervous system. a, (i) Schematic representation of the molecular lesion (red asterisk) in ubqn, the ubqn deletion \( \gamma^{\text{wg}} \Delta \text{ubqn} \) construct, the GR construct (tan box), and the deficiency spanning the genomic region (light blue box). (ii) Schematic representation of the nonsense mutation identified in ubqn, which contains an N-terminal UBL domain, four Sti1 motifs and a C-terminal UBA domain, capable of binding to the proteasome, heat shock chaperones and ubiquitinated proteins, respectively. b, Immunofluorescence staining in larval brain, ventral nerve cord and adult brain of flies expressing the GFP-tagged genomic ubqn transgene (Ubqn-GFP construct). Scale bars, 40 μm. c, Quantitative RT–PCR results showing diminished ubqn transcript expression in ubqn1 wandering third instar larvae compared to iso \( \text{y w FRT19A} \) larvae normalized against a housekeeping gene (GAPDH). \( n = 3 \) independent biological samples, and 3 PCR replicates for each biological sample. Mean ± s.e.m. ** \( P = 0.0028 \). d, Western blot of Ubqn with protein lysates from third instar larvae of iso, ubqn1; GR and ubqn1, showing diminished Ubqn levels in ubqn1 mutants. Asterisks indicate nonspecific bands. e, H&E staining in trans and frontal sections of the head of control (iso) and ubqn1 pre-pupae (P4 stage = 20 h APF grown at room temperature). The neuropil is severely reduced in mutants (shown as light pink with H&E staining), demonstrating that loss of ubqn leads to morphological defects in the developing brain. Scale bars, 100 μm. f, ERG traces from 15- and 45-day-old ey-FLP clones of iso (control), ubqn1 and ubqn1; GR raised in 12 h light–12 h dark cycle (LD) with quantification of ERG amplitudes. Phototransduction defects were observed in the ubqn1 mutant clones. \( n = 5 \) (iso 15 days old and ubqn1 45 days old), \( n = 6 \) (iso 45 days old and ubqn1; GR 45 days old) \( n = 7 \) (ubqn1 15 days old), \( n = 4 \) (ubqn1; GR 15 days old) flies. Mean ± s.e.m. NS, not significant; ** \( P = 0.0057 \), **** \( P < 0.0001 \). For all panels except e, three independent experiments were performed with similar results obtained. For e, two independent experiments were performed with similar results obtained. All statistics were determined by two-sided Student’s t-test. Statistics source data for c and f can be found in Supplementary Table 9.
To determine whether Ubqn plays a role during early development, we removed it in the female germline using the ovo method. Females with homozygous ubqn germline clones rarely laid eggs and these eggs did not develop (Supplementary Fig. 1d), showing that Ubqn is essential for oogenesis. Hence, development of ubqn mutants to the pre-pupal stage probably relies on maternally contributed Ubqn protein. We also noted developmental defects in the pre-pupae before death. Analysis of haematoxylin and eosin (H&E)-stained pre-pupae sections showed severely reduced neuropil in ubqn mutant nervous systems (Fig. 1e). Mutants also exhibited incomplete limb eversion and loss of head fat body (Supplementary Fig. 1e,f). These data show that Ubqn is required for proper development of the nervous system as well as other tissues.

Loss of Ubqn leads to age-dependent neuronal and glial degeneration. To assess the requirement of Ubqn in the ageing fly nervous system, we generated mosaic eye clones using the FLP–FRT system and recorded electroretinograms (ERGs) in mutant photoreceptors. ERG amplitudes were slightly reduced in 15-day-old flies raised in a 12 h light–12 h dark cycle. However, 45-day-old flies exhibited a severe loss of ERG amplitude (Fig. 1f). To assess whether the phototransduction defect exhibited by ubqn clones is accompanied by structural defects, we used transmission electron microscopy (TEM) of 1-, 15- and 30-day-old mutant eye clones raised in a 12 h light–12 h dark cycle. At day 1, mutant retinaiain exhibited subtle defects; however, photoreceptor numbers were comparable to controls (Fig. 2a; Supplementary Fig. 2a). At day 15, the mutant retinae displayed vacuolization, electron dense aggregates and photoreceptor loss (Fig. 2a; Supplementary Fig. 2a). Finally, at day 30, the mutant retinae exhibited numerous degenerative features, including photoreceptor loss, vacuolization, rhabdome loss, split rhabdomeres, glial death, electron dense aggregate accumulation and mitochondria accumulation (Fig. 2a–c; Supplementary Fig. 2c). Consistent with findings in the retinae, laminae from 30-day-old flies revealed that mutant synaptic terminals were severely affected, as they were enlarged fivefold, which was accompanied by photoreceptor terminal loss (Supplementary Fig. 2d). Mutant flies that were reared under constant darkness also displayed decreased ERG amplitude and neurodegeneration (Supplementary Fig. 2e,f), indicating that neurodegeneration caused by Ubqn loss is activity independent. These data show that Ubqn loss causes a slow and progressive demise of neurons, synaptic terminals and glia.

Loss of Ubqn leads to ER expansion and proteostasis defects. Ubiquilins are implicated in the regulation of ER-associated degradation (ERAD) in mammalian cells. To test the involvement of Ubqn in ER proteostasis in vivo, we examined ER abundance and morphology in ubqn clones in larval salivary glands. ubqn clones displayed significantly expanded ER, as indicated by a strong increase in the levels of the ubiquitously expressed ER marker sqh-EYFP-ER (Supplementary Fig. 3a). Similarly, TEM revealed an approximately twofold increase in the rough ER density in retinae of 1-day-old ubqn flies (Fig. 2d).

An ER expansion phenotype was previously shown in flies lacking the fly homologue of vesicle-associated membrane protein-associated protein B (VAPB; also known as ALS8). These flies also accumulated membrane proteins such as Chaoptin in the ER of a few adult neurons. Similarly, neuron-specific knockdown of Ubqn caused cytoplasmic accumulation of Chaoptin (Supplementary Fig. 3b), suggesting that Ubqn loss leads to defective trafficking and clearance of Chaoptin and probably other membrane-bound proteins.

ER expansion, coupled with defective ER protein trafficking in ubqn mutants could also promote ER stress and the unfolded protein response (UPR). ubqn mutants showed a significant increase in levels of an ER chaperone, BiP (also known as Hsc3) (Supplementary Fig. 3c). We examined the activation of two UPR pathways: inositol-requiring protein 1α (IRE1α) and protein kinase RNA-like ER kinase (PERK). Ubqn did not affect the IRE1α pathway (Supplementary Fig. 3d,e). However, ubqn mutants displayed an increase in phosphorylation of eIF2α, a component of the PERK pathway that is phosphorylated during ER stress (Fig. 2c; Supplementary Fig. 3f). These data indicate that Ubqn is required for proper ER morphology and function and that its loss causes ER stress and PERK-mediated UPR activation.

Loss of Ubqn affects mTOR signalling and promotes autophagy induction. ER stress and the UPR may mitigate proteostatic stress by enhancing autophagy through mTOR signalling, a major autophagy regulator. To determine whether Ubqn loss alters mTOR activity, we examined the phosphorylation status of direct targets of mTOR complexes, mTORC2 and mTORC1. Ubqn loss led to diminished phosphorylation of the mTORC2 target, Akt, as well as mTORC1 targets, S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein (4E-BP) (Fig. 3a). Hence, mTOR signalling is decreased following Ubqn loss.

To determine whether reduced mTORC1 activity in ubqn mutants affects autophagy induction, we assessed the levels of two autophagic markers, Atg1 and Atg8. The Atg1–ULK1 kinase complex initiates autophagy and is inhibited by mTORC1-mediated phosphorylation. ubqn clones of larval fat body showed a significant increase in Atg1-GFP punctae (Fig. 3b), indicating that there was increased autophagy induction, consistent with reduced mTORC1 activity. We monitored autophagic vesicles using mCherry-Atg8 or GFP-Atg8 reporters and observed a marked increase in both mCherry and GFP punctae in ubqn clones in larval fat body (Fig. 3c; Supplementary Fig. 4a). TEM data of 15-day-old photoreceptors confirmed that Ubqn loss causes a robust increase in the number of autophagosomes, amphisomes, autolysosomes and lysosomes (Fig. 3d; Supplementary Fig. 4b). Therefore, Ubqn loss leads to reduced mTOR signalling and enhanced autophagy induction.

Ubqn is required to maintain autophagic flux. To determine whether autophagic flux is affected in ubqn mutants, we assessed the levels of two autophagic markers, Atg1 and Atg8. The Atg1–ULK1 kinase complex initiates autophagy and is inhibited by mTORC1-mediated phosphorylation. ubqn clones of larval fat body showed a significant increase in phosphorylation of eIF2α, a component of the PERK pathway that is phosphorylated during ER stress (Fig. 3d; Supplementary Fig. 4a). These data show that Ubqn loss leads to decreased mTOR signalling and enhanced autophagy induction.

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larvae, autophagic vesicles are readily observable due to induction of developmental autophagy. ubqn1 clones in third instar larval fat body displayed aberrant and clustered LAMP1-GFP-positive compartments (Fig. 4b). In second instar larval fat body, ubqn1 clones displayed a prominent increase in the number and intensity of LAMP1-GFP-positive compartments (Supplementary Fig. 4h). These punctae were dramatically enlarged following starvation, suggesting that there was non-degraded material accumulation in
Loss of Ubqn affects mTOR signalling and promotes autophagy induction. a, Western blot of mTOR-dependent phosphorylation of Akt, S6K and 4E-BP with fat body protein lysates from third instar larvae of ubqn1; GR (control) and ubqn1. Quantification of relative P-Akt/Akt, P-S6K/S6K and P-4E-BP/Tubulin ratios shows that ubqn1 mutants exhibit decreased mTOR activity. n = 3 biologically independent samples. Mean ± s.e.m. *P = 0.0372, **P = 0.0035 (P-S6K/S6K), ***P = 0.0047 (P-4E-BP/Tubulin). b, ubqn1 clone in fat body of early third instar larvae expressing Atg1-GFP protein trap with quantification of normalized Atg1 punctae number per μm². ubqn1 mutants exhibit increased autophagy induction. White line shows the border between ubqn1 mutant clones and surrounding wild-type cells. Scale bar, 10 μm. n = 4 biologically independent samples. Mean ± s.e.m. **P = 0.007. c, ubqn1 clone in fat body of early third instar larvae expressing UAS-mCherry-Atg8a with the Cg-GAL4 driver and quantification of normalized Atg8 punctae number per μm². ubqn1 mutants exhibit increased numbers of autophagic vesicles. White line shows the border between ubqn1 mutant clones and surrounding wild-type cells. Scale bar, 10 μm. n = 8 biologically independent samples. Mean ± s.e.m. ***P = 0.0003. d, TEM images of the retinae of 15-day-old ey-FLP clones of ubqn1; GR (control) and ubqn1 and quantification of autophagic vesicle numbers confirm the results obtained for c. Scale bars, 0.5 μm. n = 4 flies. **P = 0.001, ***P = 0.0003 (autophagosome), **P = 0.0004 (autolysosome and amphisome). e, Western blot of p62 with fat body protein lysates from third instar larvae of ubqn1; GR (control) and ubqn1 and quantification of relative p62/actin levels. ubqn1 mutants exhibit increased p62. n = 5 biologically independent samples. Mean ± s.e.m. **P = 0.0009. f, Immunofluorescence staining of p62 and phalloidin (labelling rhabdomeres in PRs) in whole eye clones of 2-day-old control (iso) and ubqn1 (showing p62 aggregrates), and quantification of p62 fluorescence intensity. Scale bars, 1 μm. n = 5 (control), n = 7 (ubqn1) biologically independent animals. Mean ± s.e.m. ***P = 0.0009. For all panels except b, three independent experiments were performed with similar results obtained. For b, two independent experiments were performed with similar results obtained. All statistics were determined by two-sided Student’s t-test (statistics source data for can be found in Supplementary Table 9).

Lysosomes. Lysosomal expansion with aberrant morphology was supported by TEM analyses of photoreceptors of ubqn1 mutant eye clones (Fig. 4c).

Lysosomes must maintain an acidic pH for the degradative activity of hydrolases such as Cathepsins. We assessed acidification of the vesicles in the endo-lysosomal pathway using LysoTracker, a
Fig. 4 | Ubq facilitates lysosomal acidification and function. a. Confocal images of fed early third instar larval fat body of y w (control) and ubqn1 expressing UAS-GFP-mCherry-Atg8a driven by Cg-GAL4 (which drives expression in the fat body, haemocytes and lymph gland) and quantification of only mCherry-expressing and GFP+mCherry-expressing punctae per section (135 μm²). These results show that ubqn1 mutants exhibit impaired autophagic flux. Scale bars, 10 μm. n = 9 (control), n = 8 (ubqn1) biologically independent samples. Mean ± s.e.m. **P = 0.006, ****P < 0.0001. b, ubqn1 clone in fat body of third instar larvae expressing UAS-LAMP1-GFP with the Cg-GAL4 driver and higher magnification of LAMP1-GFP punctae to the right. ubqn1 mutants exhibit abnormal lysosomes in the fat body. White line shows the border between ubqn1 mutant clones and surrounding wild-type cells. Scale bar, 20 μm. c, TEM images of lysosomes in 15-day-old retinae of ey-FLP clones of ubqn1; GR (control) and ubqn1. ubqn1 mutants exhibit abnormal lysosomes in the retina. Scale bar, 0.5 μm. d, Live imaging of LysoTracker (LT) dye in third instar larval fat body with ubqn1 clones and quantification of normalized LT punctae number per μm² in ubqn1 clones compared with the surrounding wild-type cells. ubqn1 mutants exhibit reduced number of acidic vesicles. White line shows the border between ubqn1 mutant clones and surrounding wild-type cells. Scale bar, 10 μm. n = 8 (control), n = 10 (ubqn1) biologically independent samples. Mean ± s.e.m. ***P = 0.0004. e, Live imaging for LT in third instar larval fat bodies of iso (control) and ubqn1 expressing UAS-LAMP1-GFP with the Cg-GAL4 driver and quantification of the normalized number of lysosomes (LAMP1-GFP punctae) per μm² and the number of lysosomes without LT per μm². ubqn1 mutants exhibit compromised lysosome acidification. Scale bars, 10 μm. n = 6 biologically independent samples. Mean ± s.e.m. ****P < 0.0001. f, Live imaging of Magic Red dye, which detects active Cathepsin B, in third instar larval fat body with ubqn1 clones and quantification of the number of punctae. ubqn1 mutants exhibit reduced Cathepsin B activity. White line shows the border between ubqn1 mutant clones and surrounding wild-type cells. Scale bar, 10 μm. n = 6 biologically independent samples. Mean ± s.e.m. **P = 0.0014. For all panels, three independent experiments were performed with similar results obtained. All statistics were determined by two-sided Student’s t-test. Statistics source data for a,d–f can be found in Supplementary Table 9.
dye that labels acidic vesicles. Mutant clones in wandering third instar larval fat body displayed a strong reduction in LysoTracker staining (Fig. 4d). However, mutant clones in second instar larval fat body displayed LysoTracker staining similar to control cells despite a robust increase in the total number of lysosomes as assessed by measuring LAMP1-GFP levels (Supplementary Fig. 4h,i). To confirm this reduced lysosomal acidification, we examined the colocalization of LAMP1-GFP with LysoTracker. The vast majority of LAMP1-positive punctae in ubqn1 mutants did not contain LysoTracker (Fig. 4e); therefore, most lysosomes are not acidified. Furthermore, ubqn1 clones displayed a severe reduction in activity of the lysosomal hydrolase Cathepsin B (Fig. 4f).

To determine whether the lysosomal phenotypes are due to alterations in the transcription factor EB (TFEB, or microphthalmia-associated transcription factor [Mitf] in flies) pathway, we examined the expression levels of several TFEB target genes. We did not observe an overall change in TFEB activity, although we observed an increase in VhaM8.9 expression (Supplementary Fig. 4j). These findings provide compelling evidence to indicate that Ubqn loss, while increasing overall autophagosome and lysosome numbers, interferes with lysosomal acidification.

Ubqn interacts and colocalizes with cytoplasmic v-ATPase subunits. To identify the binding partners of Ubqn, we performed immunoprecipitation and mass spectrometry (IP-MS) using transgenic flies that express genomically tagged Ubqn-GFP. Intriguingly, eight v-ATPase subunits were identified (Supplementary Tables 3 and 4). The v-ATPase is an ATP-dependent lysosomal proton pump that generates and maintains the low intralysosomal pH. It consists of a peripheral domain (V1), an integral membrane domain (V0) and two accessory components (Fig. 5a). The IP–MS data identified almost all V1 subunits and one V0 subunit, V100/V0a, whose hydrophobic N-terminus is oriented towards the cytoplasm, suggesting that Ubqn may function at the cytoplasmic area of the v-ATPase. We examined the subcellular localization of Ubqn in larval fat body and found that Ubqn localizes to the cytoplasm and is enriched in punctae at the lysosomal surface (Fig. 5b). Subcellular fractionation showed that Ubqn is present at low levels in lysosome-enriched fractions (Fig. 5c). In addition, Ubqn and VhaSFD, an orthologue of V1H, colocalize on the lysosomal surface (Fig. 5d,e). Our findings indicate that Ubqn physically interacts with the cytoplasmic v-ATPase subunits.

Ubqn genetically interacts with v-ATPase and mediates vesicle acidification. To assess v-ATPase levels in ubqn1 mutants, we tested V100-1 (V100) expression. We found that Ubqn loss caused a marked accumulation of fragmented V100 with no alteration in V100 transcript levels (Fig. 6a; Supplementary Fig. 4j). Loss of Ubqn did not alter levels of other v-ATPase subunits, Vha55 or VhaSFD (Supplementary Fig. 5a). To assess the functional link between the v-ATPase and Ubqn, we performed genetic interaction assays by selecting the following four subunits, which have different functions: V100/V0a (proton transport), Vha68/V1A (ATP hydrolysis), VhaSFD/V1H (regulatory) and VhaM8.9 (accessory). We examined a possible enhancement or suppression of ubqn1 pre-pupal lethality by introducing one copy of a loss-of-function allele of these v-ATPase subunits. Ubqn1 mutants with reduced levels of VhaSFD or Vha68 died earlier than ubqn1 mutants as third instar larvae, suggesting an enhancement of pre-pupal lethality (Supplementary Table 5). In contrast, ubqn1 mutants with reduced levels of VhaM8.9 or V100 developed beyond the pre-pupal stage and died as pharate adults (Fig. 6b). Based on these data, we hypothesized that a reduction in V100 or VhaM8.9 subunits of v-ATPase is sufficient to reestablish lysosomal acidification, leading to delayed developmental arrest. We examined LysoTracker staining in ubqn1 larval fat body clones that carry one copy of a v100 or vhaM8.9 loss-of-function allele. Indeed, reduced levels of V100 or VhaM8.9 caused a marked increase in LysoTracker punctae in ubqn1 mutant cells (Fig. 6c), showing a significant improvement in vesicle acidification. Reduced levels of V100 or VhaM8.9 also suppressed p62 accumulation (Fig. 6d). Hence, a reduction of two functionally related subunits of the V0 domain, VhaM8.9 and V100, suppress the lethality and impaired autophagic flux associated with an increase in aberrant V100 in ubqn1 mutants (Supplementary Fig. 5b).

To test whether fragmented V100 is inserted in the v-ATPase complex, we performed subcellular fractionation and found that it accumulates in lysosome fractions of ubqn1 mutants (Supplementary Fig. 5c). Next, we tested whether V100 overexpression phenocopies lysosomal degradation defects in ubqn1 mutants. Similar to Ubqn loss, V100 overexpression led to fragmented V100 accumulation (Supplementary Fig. 5d). Moreover, it caused p62 accumulation and lysosome alkalization, but did not alter eIF2α phosphorylation (Supplementary Fig. 5d,e). Indeed, reduced V100 levels in ubqn1 mutants did not significantly restore ER expansion and ER stress (Supplementary Fig. 5g), indicating that Ubqn regulates ER function and lysosome activity independently. Importantly, mTORC1 activity was restored when V100 levels were reduced (Supplementary Fig. 5h), indicating that v-ATPase activity regulates mTORC1 in ubqn1 mutants. These findings indicate that Ubqn regulates v-ATPase activity and lysosome function through regulating the levels of the V0 subunit, V100.

Re-acidification of lysosomes suppresses defects in autophagic flux in ubqn1 mutants. The above data suggest that restoration of the lysosomal pH alone may ameliorate the diminished autophagic flux observed in ubqn1 mutants. Poly(DL-lactic-co-glycolic acid) (PLGA) nanoparticles can target and re-acidify late endosomes and lysosomes. We used acidic nanoparticles (aNPs) to explore the effect of re-acidifying lysosomes. We fed ubqn1 mutants with aNPs composed of PLGA (Supplementary Fig. 6a,b). Nile red-loaded aNPs were successfully delivered to lysosomes in fat body after feeding larvae with aNPs (Fig. 6e). We then examined whether aNP feeding can ameliorate compromised vesicle acidification in ubqn1 clones in fat body. LysoTracker staining showed that ubqn1 mutant cells display acidic vesicles comparable to surrounding wild-type cells following aNP feeding (Fig. 6f). To assess the impact of lysosomal re-acidification by aNPs, we examined the autophagic flux in ubqn1 mutants. We tested p62 levels in third instar larvae fed with varying concentrations of aNPs and observed that adding 3 mg ml–1 aNPs to the food significantly suppressed p62 accumulation in ubqn1 larvae (Fig. 6g), showing improved autophagic flux. We did not observe a suppression of UPR activation or reduced mTORC1 activity through aNP feeding (Supplementary Fig. 6c).

Role of ubiquilin in lysosome acidification is evolutionarily conserved. To assess the conservation of Ubqn function in mammalian cells, we performed a triple knockdown of UBQLN1, UBQLN2 and UBQLN4 (UBQLNs) in Daoy medulloblastoma cells (Supplementary Fig. 7a). Consistent with our observations in ubqn1 mutant flies, the depletion of UBQLNs caused reduced mTORC1 activity, as revealed by a decrease in the phosphorylation status of the mTORC1 targets S6K and ULK1 (Fig. 7a). The depletion of UBQLNs also caused increased eIF2α phosphorylation (Supplementary Fig. 7b). Next, we examined the effect of the knockdown of UBQLNs in autophagy. We observed an increase in LC3 lipidation (LC3-II) and p62 punctae, suggesting that there was an increased induction of autophagy and/or impairment in degradation (Fig. 7b,c; Supplementary Fig. 7c). Treatment with the lysosomal inhibitor bafilomycin A1 increased both p62 and LC3-II levels in cells depleted in UBQLNs more strongly than in the control cells (Fig. 7b,c). To test lysosomal degradation, we examined lysosomal

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acidification and found that the loss of UBQLNs led to reduced LysoTracker staining similar to the ubqn1 mutants (Fig. 7d), suggesting that there was impaired autophagic degradation. Consistent with the fly data, depletion of UBQLNs in human cells also caused an accumulation of fragmented V0a1, the V100 homologue, but did not alter the levels of other v-ATPase subunits (Fig. 7e).

In addition, HEK293 cells in which UBQLNs were knocked out via CRISPR–Cas9 also showed increased autophagy induction, impaired autophagic flux, aberrant lysosomes and fragmented V0a1 accumulation (Supplementary Fig. 7d–f). To determine whether the physical interaction between fly Ubqn and V100 identified through IP–MS is conserved, we performed co-immunoprecipitation experiments in HEK293T cells. Under basal conditions, we were unable to co-immunoprecipitate V0a1 using FLAG-tagged UBQLN1 or UBQLN2 unless a crosslinking reagent, dithiobis(succinimidyl propionate) (DSP), was included, indicating...
ubq1n1 mutants exhibit upregulation of fragmented V100. n=5 biologically independent samples. Mean ± s.e.m. **P=0.0091. b, Images of ubq1n1 pre-pupa, ubq1n1; v100+/+ and ubq1n1; vhaM8.9/y-HA-1890/+ pharate adults. ubq1n1 lethality is suppressed by the reduction in v100 or vhaM8.9 levels. c, Live imaging of LT dye in third instar larval fat body with ubq1n1 clones of v100+/+ and vhaM8.9/y-HA-1890/+ and quantitative analyses of normalized LT-positive punctae number per μm². Reduced v100 or vhaM8.9 levels suppressed defective lysosome acidification. White lines show the border between ubq1n1 mutant clones and surrounding wild-type cells. n=16 (wild-type cell), n=11 (ubq1n1 cell), n=10 (ubq1n1; v100+/+ cell), and n=12 (ubq1n1; vhaM8.9/y-HA-1890/+ cell) independent samples. Mean ± s.e.m. ns, not significant; **P=0.0053 (ubq1n1 versus ubq1n1; v100+/+). **P=0.0013 (ubq1n1 versus ubq1n1; vhaM8.9/y-HA-1890/+). ****P<0.0001. Scale bars, 10 μm. d, Western blot of p62 with fat body protein lysates from third instar larvae of ubq1n1; GR (control), ubq1n1, ubq1n1; v100+/+, and ubq1n1; vhaM8.9/y-HA-1890/+ and quantification of normalized p62/actin levels. n=5 biologically independent samples. Mean ± s.e.m. **P=0.001. e, Live imaging of Nile red in the fat body of third instar larvae expressing UAS-LAMP1-GFP driven by the Cg-GAL4 driver after feeding the larvae with 1 mg ml⁻¹ aNPs loaded with Nile red for 3 h. aNPs are able to be delivered to lysosomes. Scale bars, 10 μm. f, Live imaging of LT in third instar larval fat body with ubq1n1 clones after feeding the larvae with 3 mg ml⁻¹ aNPs for 3 h. aNPs acidify vesicles in ubq1n1 mutants. White line shows the border between ubq1n1 mutant clones and surrounding wild-type cells. Scale bar, 10 μm.

Fig. 6 | Ubq1n1 genetically interacts with v-ATPase and mediates vesicle acidification. a, Western blot of V100 with protein lysates from third instar larvae of ubq1n1; GR (control) and ubq1n1 and quantification of full length (fL) and fragmented (fg) V100 protein levels. ubq1n1 mutants exhibit upregulation of fragmented V100. n=5 biologically independent samples. Mean ± s.e.m. **P=0.0009. For all panels except e-g, three independent experiments were performed with similar results obtained. For panels e-g, two independent experiments were performed with similar results obtained. All statistics were determined by two-sided Student’s t-test. Statistics source data for a, c, d and g can be found in Supplementary Table 9.
a weak and/or transient physical association of ubiquilins with V0a1 (Supplementary Fig. 7g).

In contrast to the roles of UBQLN1 and UBQLN2, a role for UBQLN4 in proteostasis has not been well established. We examined the effect of UBQLN4 knockdown on UPR activation and autophagic degradation in Daoy cells. UBQLN4 depletion did not significantly alter eIF2α phosphorylation (Supplementary Fig. 8a,b). However, UBQLN4 depletion caused p62 accumulation and reduced LysoTracker staining similar to triple knockdown of the UBQLNs (Supplementary Fig. 8c,d), suggesting an important role for UBQLN4 in the regulation of lysosomal degradation. These data show that the role of ubiquilins in the regulation of autophagic

Fig. 7 | Role of ubiquilin in lysosomal acidification is conserved in human neuronal cells. a, Western blot of P-S6K (T389), S6K, P-ULK1 (S757) and ULK1 from Daoy whole cell lysates transfected with control siRNA (siCtrl) or siRNA to knock down UBQLN1, UBQLN2 and UBQLN4 (siUBQLNs), and quantification of phosphorylated/total protein ratios. Triple knockdown reduces mTORC1 activity. n = 3 biologically independent samples. Mean ± s.e.m. **P = 0.0017 (P-S6K/S6K), ***P = 0.0099 (P-ULK1/ULK1). b, Western blot of LC3-I and LC3-II with Daoy whole cell lysates cells transfected with siCtrl or siUBQLNs with or without 4 h of bafilomycin A1 (BafA1) treatment, and quantification of LC3-II/LC3-I ratio. DMSO, dimethylsulfoxide. n = 5 biologically independent samples. Mean ± s.e.m. ***P = 0.0008, ****P < 0.0001. c, Immunofluorescence staining of p62 and DAPI in Daoy cells transfected with siCtrl or siUBQLNs with or without 4 h of BafA1 treatment, and quantification of normalized mean p62 fluorescence intensity per cell. n = 5 images (~40 cells). Mean ± s.e.m. **P = 0.0015, ****P < 0.0001. Scale bars, 10 μm. d, Live imaging for LT in Daoy cells transfected with siCtrl or siUBQLNs, and quantification of normalized mean LT fluorescence intensity per cell. n = 10 (siCtrl) and n = 13 (siUBQLNs) images (~100 cells). Mean ± s.e.m. ****P < 0.0001. Scale bars, 10 μm. e, Western blot of LAMP1 and v-ATPase subunits (V0a1, V1B2, M8.9, V1C1 and V1H) from with Daoy whole cell lysates transfected with siCtrl or siUBQLNs. For all panels, three independent experiments were performed with similar results obtained. All statistics were determined by two-sided Student’s t-test. Statistics source data for a–d can be found in Supplementary Table 9.
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flux and v-ATPase-mediated lysosome acidification is conserved between Drosophila and humans.

**Ubiquilin and ALS.** Previously, ALS-linked mutations in UBQLN2 were shown cause defective chaperone binding and protein aggregate clearance, independent of autophagy\(^2\). The ALS-linked mutations in UBQLN2 are dominant and cluster in the PXXP domain\(^2\) that is not conserved in fly Ubqn even though the proteins are highly conserved. Hence, we cannot introduce ALS-linked mutations and replace the conserved residues in fly Ubqn. We therefore resorted to the ubiquitous overexpression of UBQLN2\(^{WT}\) in wild-type flies and showed that it is toxic as it caused lethality. We overexpressed both UBQLN2\(^{WT}\) and the ALS variant UBQLN2\(^{P497H}\) in fat body to assess their effects on ER stress and lysosomal degradation. UBQLN2\(^{P497H}\) did not alter elf2\(\alpha\) phosphorylation but caused defects consistent with the effects of ubiquilin loss observed in flies and human cells. That is, the accumulation of p62, fragmented V100 and reduced LysoTracker staining (Fig. 8a–e). These findings suggest that UBQLN2\(^{P497H}\) may act as a dominant negative mutation and that it affects the interaction of ubiquilin with v-ATPase to impair lysosome activity.

**Discussion**

Here, we showed that Ubqn interacts with the v-ATPase and is required for lysosomal acidification. Lysosomal re-acidification by aNPs ameliorated the impaired autophagic flux in ubqn\(^1\) mutants. The role of Ubqn in the regulation of autophagy and lysosomal activity is conserved in human cells. Our data provide compelling evidence to indicate that Ubqn affects two key players in proteostasis: ER quality control and autophagy by regulating lysosomal acidification (Fig. 8f).

**Role of ubiquilin in ERAD and lysosomal degradation.** Ubiquilins are largely studied in the context of protein quality control through proteasomal degradation. Previous findings have documented delayed degradation of membrane-bound and luminal ERAD
substrates following UBQLN1 or UBQLN2 depletion in vitro. We found that Ubqn loss in vivo caused membrane protein accumulation and a marked increase in ER volume coupled with ER stress and induction of the PERK branch of the UPR pathway. Although it is plausible that chronic ER stress is detrimental, some data argue that induction of low-level ER stress has protective roles. We propose that activation of the PERK pathway may play a role in increased autophagy induction and autophagosome formation in ubqn mutants, as previous reports have shown that ER stress can trigger autophagy to induce compensatory degradation.

Our findings show that Ubqn loss leads to lysosome alkalization and diminished autophagic flux. Hence, we argue that lysosomal dysfunction in ubqn mutants causes a blockade in autophagic flux despite increased autophagy induction.

Ubiquilins regulate mTOR activity, autophagy and lysosomal acidification. UBQLN1 and UBQLN2 were previously shown to have a role in autophagy. It has been argued that UBQLN1 and UBQLN2 knockdown affects maturational defects observed in ubqn1 mutants. Indeed, restoration of v-ATPase activity by manipulating V100 subunit levels in ubqn1 mutants indicates that Ubqn is involved in the regulation of lysosomal activity.

In summary, we have identified a previously undocumented function for Ubqn in the regulation of lysosomal activity.

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Author contributions

M.S. conceived the project, designed and performed the majority of the experiments, and analysed the data. M.S. and Z.Z. performed the TEM and histology experiments. E.W. and A.G.M. synthesized the aNPs. M.S., G.L. and D.M. performed the cell culture experiments. M.S. and H.J.B. wrote the manuscript. H.J.B supervised the project.

Competing interests

The authors declare no competing interests.

Additional information

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Methods

Live imaging of LysoTracker and Magic Red staining. Second or third instar Drosophila larvae were dissected in PBS and then stained with 1x LysoTracker Red DND-99 (L7528, ThermoFisher) or 1x Magic Red cresyl violet-(RR)2 DND-99 (L7528, ThermoFisher) for 3 min on a shaker. After quickly washing with PBS, samples were mounted in 80% glycerol and were imaged using a Zeiss LSM880 confocal microscope (Carl Zeiss).

Preparation of aNPs. PLGA nanoparticles were synthesized as previously published protocols22,23. PLGA (Resomer 503H, Sigma) was dissolved in dichloromethane (DCM) to form a solution of 10 mg mL⁻¹. Ten millilitres of this solution was added to 60 mL of 2-wt% PVA (87–90% hydrolysed; MW 30,000–70,000, Sigma) and sonicated for 2.5 min. The resulting oil-in-water emulsion was stirred for 4 h to remove any residual DCM. The aNPs were recovered via centrifugation at 10,000 × g for 25 min, then washed twice with refrigerated Millipore water. The aNPs were flash frozen in liquid nitrogen and dried for 48 h. Nile red-loaded aNPs were recovered via centrifugation. After two subsequent washing steps, the recovered aNPs were flash frozen and vacuum dried for 48 h. Characteristics of the aNPs can be measured by TEM analyses.

Freshly prepared aNPs were resuspended at a concentration of 10 mg mL⁻¹ in 0.1 M 2-morpholinoethanesulfonic acid (MES) buffer (pH 5.5).

TEM analyses. aNPs were flash frozen in liquid nitrogen and dried for 48 h. Nile red-loaded aNPs were resuspended with MES buffer (pH 5.5) and sonicated for 2.5 min. The resulting oil-in-water emulsion was stirred for 4 h to remove any residual DCM. The aNPs were recovered via centrifugation. After coating, the aNPs were covalently coated in BS (Sigma) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (ThermoFisher) and N-hydroxy succinimide (NHS) (ThermoFisher). EDC and NHS were added in a 1:1 molar excess (relative to PLGA) of EDC and was added and the solution was stirred for 10 min, after which 2x molar excess of NHS was added. After stirring for 2 h, the pH of the solution was titrated to 7.2 using sodium bicarbonate (Sigma) and an equimolar amount of BS (Sigma) with respect to the labile amino groups of the aNPs by centrifugation. For the following steps, the recovered aNPs were flash frozen and vacuum dried for 48 h. Coating of the aNPs was performed by centrifugation at 10,000 × g for 30 min, resuspended in 1:1 vol/vol in 85.6% Nycodenz, and placed on the bottom of an Ultraclear (Beckman Coulter) 15 ml ultracentrifuge tube containing a discontinuous gradient of Nycodenz (Beckman Coulter, WI, USA). The gradient was made up of a discontinuous gradient of Nycodenz from bottom to top: 32.8%, 26.3% and 19.8% Nycodenz. The sediment of the second centrifugation was washed at 17,000 × g, 10 min, resuspended in 1:1 vol/vol in 85.6% Nycodenz, and placed on the bottom of an Ultraclear (Beckman Coulter) 15 ml ultracentrifuge tube containing a discontinuous gradient of Nycodenz (Beckman Coulter, WI, USA). The gradient was made up of a discontinuous gradient of Nycodenz from bottom to top: 32.8%, 26.3% and 19.8% Nycodenz. The centrifugation was performed for 1 h in an SW 40 Ti rotor (Beckman) at 141,000 × g. Top layers containing lysosomes were collected and diluted in assay buffer and centrifuged at 37,000 × g for 15 min. The pellet was resuspended in assay buffer and centrifuged at 37,000 × g for 15 min. The pellet was resuspended in assay buffer and centrifuged at 37,000 × g for 15 min. The pellet was resuspended in assay buffer and centrifuged at 37,000 × g for 15 min. The pellet was resuspended in assay buffer and centrifuged at 37,000 × g for 15 min.

Subcellular fractionation. Lysosomes were enriched by centrifugation from a large pool of L3 larvae in a discontinuous Nycodenz density gradient, as described previously24,25 but with modifications. In brief, homogenate was prepared in 0.25 M sucrose (pH 7.2) and centrifuged in succession at 4,800 × g for 5 min and 17,000 × g for 10 min. The sediment of the second centrifugation was washed at 17,000 × g, 10 min, resuspended in 1:1 vol/vol in 85.6% Nycodenz, and placed on the bottom of an Ultraclear (Beckman Coulter) 15 ml ultracentrifuge tube containing a discontinuous gradient of Nycodenz (Beckman Coulter, WI, USA). The gradient was made up of a discontinuous gradient of Nycodenz from bottom to top: 32.8%, 26.3% and 19.8% Nycodenz. The centrifugation was performed for 1 h in an SW 40 Ti rotor (Beckman) at 141,000 × g. Top layers containing lysosomes were collected and diluted in assay buffer and centrifuged at 37,000 × g for 15 min. The pellet was resuspended in assay buffer and a standard western blotting protocol was then followed.

Drosophila antibody. Immunofluorescence. The following primary antibodies were used: rabbit anti-p62/ret(2)p (1:1,000; gift from S. Zhang); chicken anti-GFP (1:500; Abcam, ab13970); rat anti-ELAV (1:500; Developmental Studies Hybridoma Bank (DSHB), #7CA10); mouse anti-Chaoptin (1:100; DSHB, 24B10); and rabbit anti-Akt (1:200; gift from M. Guo). Detailed primary antibody information can be found in Supplementary Table 7. The following secondary antibodies were used: Alexa 488-, Cy3- or Cy5-conjugated secondary antibodies (1:500; Jackson ImmunoResearch). Alexa 488-conjugated Phalloidin (Invitrogen) was used at 1:250 dilution.

Western blotting. The following primary antibodies were used: rabbit anti-Ubqn (1:2,000; gift from M. Guo); mouse anti-actin (1:10,000; ICN Biomedicals, C4); mouse anti-α-Tubulin (1:10,000; Sigma, T9026); rabbit anti-P-eIF2α (S51) (1:2,000; Cell Signaling Technology, CST, 9721); rabbit anti-P-eIF2α (eIF2S1) (1:1,000; Abcam ab26197); rabbit anti-GFP (1:7,500; Invitrogen, A-11212); rabbit anti-DiirRed (1:2,000; Clontech, 632496); rabbit anti-Phospho-Phospho-Dcpl-Akt (Ser505) (1:1,000; CST, 4054); rabbit Akt (pan) (1:1,000; CST, 4691, C67E7); rabbit anti-Phospho-Phospho-S6K (Thr389) (1:1,000; CST, 9209); rabbit anti-S6K (1:1,000; SCBT, sc-9027); rabbit anti-Phospho-4E-BP (Thr37/46) (1:1,000; CST, 2853); rabbit anti-p62/ret(2)p (1:2,000; gift from S. Zhang); guinea pig anti-Vha100 (1:1,000); mouse anti-CTSL (1:1,000; R&D Systems, MAB2291); rabbit anti-ATP6V1B2/ Vha55 (1:1,000; CST, 14671); guinea pig anti-Bip/Hev3 (1:1,000; gift from D. Ryoo); rabbit anti-Phospho-Dcpl-Atg8 (1:1,000; gift from L. You); mouse anti-Lamin C (1:1,000; DSHB, LC28.26). Detailed primary antibody information can be found in Supplementary Table 7. Secondary antibodies, IRdye 680RD and IRdye 800CW (Li-COR Biosciences), were used at 1:1,000, and horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch) were used at 1:5,000. Images have been cropped for presentation. Full size images are presented in Supplementary Fig. 9.

ERG assay. For ERG recordings, y w ubirni FRT19A/FM7,C; Kr-Gal4, UAS-GFP flies were crossed to y w (+) w (cl) FRT19A/Da[y+]; ey-GFP to generate flies with mutant clones in the eyes, and flies were aged at 25°C under a 12 h light–12 h dark cycle or in 24 h of darkness. ERG recordings were performed as previously described26. At least five flies were examined for each genotype and timepoint.

RNA extraction and quantitative real-time PCR. Total RNA was isolated from 10 third instar larvae using TRIzol (Life Technologies). Reverse transcription was performed using iScript Reverse Transcription Supermix (Bio-Rad). RT–PCR was performed using iQ SYBR Green Supermix (Bio-Rad) and a CFX96 Touch Real-Time PCR Detection System. RT–PCR was performed with 3–6 PCR replicates for each biological sample, 3–4 biological replicates. Primers used for the RT–PCR experiments are listed in the Supplementary Table 8.

IP–MS analyses. The larvae or adult head pellet was lysed in 3 volumes of NETN buffer (50 mM Tris (pH 7.3), 170 mM NaCl, 1 mM EDTA, 0.5% NP-40) using...
Cells were grown and maintained as described previously9. For the inhibitor treatments, HEK293 cells were treated with bafilomycin A1 (200 nM for 4 h) before collection or fixation. Daoy cells were maintained in DMEM medium supplemented with 10% FBS, 1 M Tris (pH 7.5) to a final concentration of 20 mM for 15 min. Crosslinked cells were transiently transfected into HEK293T cells using Lipofectamine 3000 (p4455 FLAG-hPLIC-2; Addgene, 8661) cDNA for 6 h and then lysed using Tris-Cl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 1 mM NaVO4, 10% glycerol and Roche protease inhibitor mix) on ice. HEK293 cells were homogenized in Sds lysis buffer (2% SDS, 50 mM Tris-HCl (pH7.4), 2 mM EDTA) on ice. Isolated lysates heated in an appropriate volume of Laemmli buffer were loaded into gels, separated by SDS–PAGE, and transferred to nitrocellulose membranes (Bio-Rad). Primary antibodies used in this study were as follows: rabbit anti-GAPDH (1:10,000; CST, 4949); rabbit anti-UBQLN1 (1:1,000; CST, 58559); rabbit anti-UBQLN2 (1:1,000; CST, 9091); rabbit anti-LAMP2 (1:2,000; SCBT, H4B4); rabbit anti-LC3B (1:50,000; ThermoFisher, DR11); rabbit anti-DDK (1:5,000; MBL, PM045); rabbit anti-LAMPI (1:1,000; CST, 14526); rabbit anti-ATP6V1H (1:1,000; CST, 14526); rabbit anti-ATP6V1B2 (1:1,000; CST, 14526); mouse anti-UBQLN1 (1:500; CST, sc-271077); mouse anti-ATP6V1D (1:500; SCBT, sc-390384); mouse anti-ATP6V1H (1:500; SCBT, sc-166227); rabbit anti-Renin R/M8.9 (1:2,000; Novus Biologicals, NBPI-90820); mouse anti-FLAG M2 (1:1,000; Sigma, F1804). Detailed primary antibody information can be found in Supplementary Table 7. Secondary antibodies, IRdye 680RD and IRdye 800CW (Li-COR Biosciences), were used at 1:10,000 dilution, and HRP-conjugated secondary antibodies (Jackson Immunoresearch) were used at 1:15,000 dilution. Images have been cropped for presentation. Full size images are presented in Supplementary Fig. 9.

Nanoparticle characterization. The size and zeta potential of nPs were measured using a Zetasizer Nano: Malvern Zen 3600 Zetasizer. Particles (0.1 mg) were added to filtered Millipore water (1 ml), allowed to swell for 6 h, and then measured. For BSA loading, a BSA per mg of anP was measured using a Micro BCA Protein Assay kit (ThermoFisher). Briefly, nanoparticles were suspended in Millipore water at a concentration of 0.1 mg/ml and added to an equal volume of working solution. After 2 h of incubation at 37°C, the suspension was centrifuged, and the supernatant removed for analysis. For the analysis, BSA-coated PLGA nanoparticles were compared to blank uncoated PLGA nanoparticles, and BSA-coated Nile red-loaded PLGA nanoparticles were compared to Nile red-loaded uncoated nanoparticles, as some of the Nile red was released from the nanoparticles during the incubation period. For scanning electron microscopy analyses, vacuum dried nanoparticles were coated with 10 nm of gold using a Denton Desk V Sputter system. Particles were imaged using a FEI Quanta 400 ESEM FEG at 10 kV.

Statistics and reproducibility. All quantitative data were derived from multiple independent experiments. Each exact n value is indicated in the corresponding figure legend. Two-tailed Student's t-test was used to analyse data, and a P value of <0.05 was considered significant. No statistical method was used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments. Experiments in the manuscript were performed at least three times except for those presented in Figs. 1e, 3f, 5c, 7e–g, and Supplementary Figs. 1–6. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Code availability
There is no custom code used in this study.

Data availability
The MS proteomics data can be accessed from the ProteomeXchange Consortium via the MassIVE repository (MSV000083259) under accession code PXD012104.
The authors declare that the main data supporting the findings of this study are available within the article and its Supplementary Information files. Source data for Figs. 1c,f, 2a–e, 3b–f, 4a,d–l, 5a,e,d,g, 7a–d, and Supplementary Figs. 1f, 2a,d,e, 3a–c, 4b,c,g,j, 5a,e,f, 7b,c, and 8b–d have been provided as Supplementary Table 9. All other data that support the findings of this study are available from the corresponding author upon reasonable request.

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Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

| Item                                                                 | Confirmed |
|----------------------------------------------------------------------|-----------|
| The exact sample size \( n \) for each experimental group/condition, given as a discrete number and unit of measurement | Yes       |
| An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly | Yes       |
| The statistical test(s) used AND whether they are one- or two-sided | Yes       |
| Only common tests should be described solely by name; describe more complex techniques in the Methods section. | No        |
| A description of all covariates tested                              | Yes       |
| A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons | Yes       |
| A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) | Yes       |
| For null hypothesis testing, the test statistic (e.g. \( t \), \( t \), \( r \)) with confidence intervals, effect sizes, degrees of freedom and \( P \) value noted | Yes       |
| Give \( P \) values as exact values whenever suitable. | Yes       |
| For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings | Yes       |
| For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes | Yes       |
| Estimates of effect sizes (e.g. Cohen's \( d \), Pearson's \( r \)), indicating how they were calculated | Yes       |
| Clearly defined error bars                                           | Yes       |
| State explicitly what error bars represent (e.g. SD, SE, CI)         | Yes       |

Software and code

Policy information about availability of computer code

| Data collection | Fluorescent images were acquired with Zeiss LSM710 or Zeiss LSM880 confocal microscopes and Zen software (Zeiss). |
|-----------------|---------------------------------------------------------------------------------------------------------------|
| Data analysis   | For image analysis, Image J or LI-COR Image Studio softwares were used. Quantitative data were analyzed and processed using MS Excel and GraphPad Prism 7.04. Xcalibur software (Thermo Scientific) and Proteome Discoverer 1.4 interface (Thermo Scientific) with Mascot algorithm (Mascot 2.4, Matrix Science) were used for mass spectrometry analysis. |

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The MS proteomics data can be accessed from the ProteomeXchange Consortium via the MassIVE repository (MSV000083259) under accession code PXD012104.
The authors declare that the main data supporting the findings of this study are available within the article and its Supplementary Information files. Source data for Figures 1c, 1f, 2c-f, 4a, 4d-f, 5e, 6a, 6c-d, 6g, 7a-d, S1f, S2a, S2d-e, S3a-c, S4b-c, S4g, S4j, S5a, S5e-f, S7b-c, and S8b-d have been provided as Supplementary Table 9. All other data that support the findings of this study are available from the corresponding author upon reasonable request.

Field-specific reporting

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- Life sciences
- Behavioural & social sciences
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample sizes were determined based on published studies in the field or our previous experiences. No statistics was used to predetermine the sample size. |
| Data exclusions | No data were excluded. |
| Replication | Experiments in the manuscript were performed at least three times except for Figures 1e, 3b, 5c, 6e-g, S1c, S1f, S3d-f, S4d, S4e-f, S5f, S5h, S6c, S7g, S8d, and larval IP/MS experiments (in Supplementary Tables 3-4) which were performed twice and adult head IP/MS experiment (in Supplementary Tables 3-4) which was performed once. All attempts at replication were successful. |
| Randomization | Samples are defined by their unique genotypes. Therefore, no sample randomization was performed. |
| Blinding | The investigators were not blinded for group allocation as the mutants displayed obvious phenotypes and clonal analysis marks mutants. |

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a

- Unique biological materials
- Antibodies
- Eukaryotic cell lines
- Palaeontology
- Animals and other organisms
- Human research participants

Methods

n/a

- ChIP-seq
- Flow cytometry
- MRI-based neuroimaging

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

All unique materials used are readily available from the authors upon reasonable request. Fly stocks generated in this work will be deposited in BDSC upon publication.

Antibodies

Antibodies used

Primary antibodies used in fly experiments:
- Rabbit anti-p62/ref(2)p (gift from Dr. Sheng Zhang); Chicken anti-GFP (Abcam, ab13970); Rat anti-ELAV (DSHB: 7E8A10); Mouse anti-Chaoptin (DSHB: 24810); Rabbit anti-Ubqn (gift from Dr. Ming Guo); Mouse anti-actin (ICN Biomedicals: C4); Mouse anti-α-Tubulin (Sigma T9026); Rabbit anti-P-eIF2α (S51) (CST #9721); Rabbit anti-eIF2α (eIF251) (Abcam ab26197); Rabbit anti-GFP (Invitrogen RA-11122); Rabbit anti-Phospho-Drosophila-Akt (Ser505) (CST #4054); Rabbit Akt (pan) (CST #4691); Rabbit anti-Phospho-Drosophila-S6K (Thr398) (CST #9209); Rabbit anti-S6K (SCBT sc-9027); Rabbit anti-Phospho-4E-BP (Thr37/46) (CST #2855); Mouse anti-CTS1 (R&D Systems RMAB22591); Guinea pig anti-Bip/Hsc3 (gift from Dr. Don Ryoo); Rabbit anti-Drosophila Atg8 (gift from Dr. Linda Partridge); Mouse anti-Lamin C (DSHB, LC28.26); Guinea pig anti-Vha100 (generated in the Bellen Lab).

Primary antibodies used in human cell culture experiments:
Rabbit anti-p62 [MBL: PM045]; Rabbit anti-GAPDH [CST #85509]; Rabbit anti-UBQLN1 [CST #14526]; Rabbit anti-UBQLN2 [CST #85509]; Rabbit anti-UBQLN4 (Abcam ab106443); Rabbit anti-Phospho S6K(T389) [CST #9205]; Rabbit anti-S6K [SCBT sc-9027]; Rabbit anti-Phospho ULK1(S757) [CST #6888]; Rabbit anti-LAMP1 [SCBT sc-7580]; Rabbit anti-LAMP2 [SCBT: H4B4]; Rabbit anti-LC3B [CST: D11]; Rabbit anti-ATP6V0A1 (Novus Biologicals: NB1-89342); Rabbit anti-ATP6V1B2 [CST #14617]; Mouse anti-LAMP2 [SCBT: sc-9091]; Mouse anti-ATP6V1D1 [SCBT: sc-393322]; Mouse anti-ATP6V1H [SCBT: sc-166227]; Rabbit anti-Renin R/M8.9 [Novus Biologicals: NB1-90820]; Mouse anti-FLAG M2 (Sigma: F1804)

Secondary antibodies used:
For IF: Alexa 488, and Cy3 or Cy5 conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) and Alexa 488-conjugated Phalloidin (Invitrogen)
For WB: IRdye 680RD and IRdye 800CW (Li-COR Biosciences) and HRP-conjugated secondary antibodies (Jackson ImmunoResearch)

All antibody dilutions are listed in Methods.

Validation
All antibodies were validated by the manufacturer or by previously published studies to be suitable for immunofluorescence and/or western blotting in flies or human cell lines. Antibody validation information is listed in Supplementary Table 7.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
Dasy and HEK293T cell lines were from Dr. Huda Zoghbi and HEK293 UBQLN1+2+4 triple knockout cell line was from Dr. Ramanujan S. Hedge.

Authentication
Cell lines were authenticated based on their morphology and growth.

Mycoplasma contamination
Cell lines were not tested for mycoplasma contamination.

Commonly misidentified lines
No cell lines used in this study were found in the database of commonly misidentified cell lines that is maintained by ICLAC and NCBI Biosample.

Animals and other organisms

Policy information about studies involving animals, ARRIVE guidelines recommended for reporting animal research

Laboratory animals
Drosophila melanogaster adult flies or larvae were used depending on each experimental design.
L2 larvae: S4h-1
Early L3 larvae: 3b-c, 4a, S4a, and S4g
Wandering L3 larvae: 1b-d, 3a, 4b, 4d-f, 5b-e, 6a, 6c-g, 8a-e, 51a-c, 53a, 53c-f, 54, 55a-h, and 56c
Pre-pupa: 1e, 51e-f, and 54f
Adult flies:
1b: 2d old adult female
1f: 15d and 45d old females
2a-c, S2a-d, and S4b: 1d, 15d, and 30d old females
2d: 1d old females
3d, 4c: 15d old females
3f: 2d old adult females
S2e: 45d old females
S2f: 30d old females
S3b: 2-3d old adult females

Following strains are used in this study:
y w ubqn[1] FRT19A, y w ubqn[1] FRT19A; 20kb P[acman], ubqn[ywing2+], UAS-dUbqn, UAS-FLAG-dUbqn, y w;UAS-UBQLN1[WT], y w;UAS-UBQLN2[P497H], y w; FRT82B v100[3], and UAS-V100 were generated in the Bellen lab.
y1 w* P(nos-phiC31int.NLS) P{eyFLP}, P{Ubi-mRFP.nls}, w* P(hsFLP), P(neoFRT)19A; P(eyFLP), P(Ubi-mRFP.nls), w* P(hsFLP), P(neoFRT)19A; P(UAep-mCherry-Atg8a)2, UAS-Luciferase RNAi, UAS-Luciferase, y v1; UAS-V100 RNAi, w*; P[w+mC]=sqh-EYFP-ER3, y[1] w[1118]; P(UAep-mCherry-Atg8a)2, y[1] w*; Mi[P1-ATP6V1B2]; P(ubiquit-EGFP)1Atg1MI06808-1, P(ubiquit-EGFP)1Atg1MI06808-2, and P(ubiquit-EGFP)1Atg1MI06808-3 were obtained from Bloomington Drosophila Stock Center.
y[1118]; P(ubiquit-EGFP)1Atg1MI06808-1, P(ubiquit-EGFP)1Atg1MI06808-2, and P(ubiquit-EGFP)1Atg1MI06808-3 were obtained from Bloomington Drosophila Stock Center.

Wild animals
This study did not involve wild animals.

Field-collected samples
This study did not involve samples collected from the field.