A *C9orf72* ALS/FTD ortholog acts in endolysosomal degradation and lysosomal homeostasis

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Summary

The most common genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) is the expansion of a hexanucleotide repeat in a non-coding region of the gene *C9orf72*. We report that loss-of-function mutations in *alfa-1*, the *Caenorhabditis elegans* ortholog of *C9orf72*, cause a novel phenotypic defect: endocytosed yolk is abnormally released into the extra-embryonic space, resulting in refractile "blobs." The *alfa-1* blob phenotype is partially rescued by the expression of the human C9orf72 protein, demonstrating that *C9orf72* and *alfa-1* function similarly. We show that *alfa-1* and *R144.5*, which we identified from a genetic screen for mutants with the blob phenotype and renamed *smcr-8*, act in the degradation of endolysosomal content and subsequent lysosome reformation. The *alfa-1* abnormality in lysosomal reformation results in a general dysregulation in lysosomal homeostasis, leading to defective degradation of phagosomal and autophagosomal contents. We suggest that, like *alfa-1*, *C9orf72* functions in the degradation of endocytosed material and in the maintenance of lysosomal homeostasis. This previously undescribed function of *C9orf72* explains a variety of disparate observations concerning the effects of mutations in *C9orf72* and its homologs, including the abnormal accumulation of lysosomes and defective fusion of lysosomes to phagosomes. We suggest that aspects of the pathogenic and clinical features of ALS/FTD caused by *C9orf72* mutations, such as altered immune responses, aggregation of autophagy targets and excessive neuronal

excitation, result from a reduction in *C9orf72* gene function and consequent abnormalities in lysosomal degradation.

Introduction

Amyotrophic lateral sclerosis (ALS), a devastating motor neuron disease, shares clinical, neuropathological and genetic features with frontotemporal dementia (FTD), suggesting that the two diseases might be different manifestations of the same disorder[1,2]. Supporting this idea, a $(GGGGCC)_{>30}$ hexanucleotide repeat expansion in a non-coding region of the *C9orf72* gene is the most common genetic cause of both ALS and FTD[3,4]. Possible pathological mechanisms underlying the neurodegeneration in *C9orf72* ALS/FTD patients include loss of the normal function of C9orf72, expression of repeat-containing RNA and/or expression of dipeptide-containing proteins generated via unconventional translation[5–13].

Sequence and structural analyses led to the suggestion that *C9orf72* and its homologs in other species are related to differentially expressed in normal and neoplasia (DENN) proteins[14,15]. DENN proteins act as GDP-GTP exchange factors for Rab GTPases, small GTPase molecular switches involved in almost all endomembrane trafficking events in eukaryotes[16]. Recently, C9orf72 has been shown to affect endocytic transport, lysosomal biogenesis, and autophagy, the latter by forming a heterodimer with SMCR8 (Smith-Magenis syndrome chromosome region 8) and regulating Rab39 activity[17–21].

To better understand the normal function of C9orf72, the relationship between C9orf72 function and ALS, and possible toxic effects of drugs designed to decrease hexanucleotide repeat-containing *C9orf72* expression and function,

we have analyzed its *Caenorhabditis elegans* ortholog, ALFA-1 (ALS/FTD associated gene homolog 1)[8,15] (Fig. 1A).

Results

Mutations in the *C. elegans* ortholog of the ALS gene *C9orf72* cause defects in yolk metabolism in the embryo

Mutations in the *C. elegans* gene *alfa-1,* the ortholog of the human ALS gene *C9orf72*, lead to motor neuron degeneration and stress sensitivity[8]. We discovered a novel abnormal phenotype of embryos with mutations in *alfa-1* (Figure 1A): refractile bodies, which we refer to as “blobs”, floating in the extra-embryonic fluid (Figure 1B). These blobs are not membrane bound and do not contain DNA, indicating that they are neither cells nor fragments of cells (data not shown). We generated transgenic animals that expressed fluorescent reporters for the cholesterol-binding yolk proteins VIT-2[22], VIT-5 and VIT-6 and detected all three reporters in the blobs (Figure 1B and data not shown). *C. elegans* yolk is a lipoprotein complex generated in the intestine of the adult hermaphrodite and transported into the oocyte via receptor-mediated endocytosis. During the morphogenesis stage of embryogenesis when cells differentiate, yolk has been reported to be released from non-intestinal cells, transported into the embryonic intestine and used as an energy source during embryonic development and post-embryonic survival under adverse conditions, such as starvation[23,24].

Mutations in the oocyte’s yolk receptor gene *rme-2* lead to a decreased uptake of yolk by the oocyte[22]. A loss-of-function mutation of *rme-2* suppressed the blob phenotype of *alfa-1(ok3062)* embryos (Figures 1C and 1D) supporting our

conclusion that the blobs contain yolk. The *alfa-1* blob phenotype is not caused by an excess uptake of yolk by the oocyte or a defective uptake of yolk by the embryonic intestine, but rather by an abnormal release of yolk from yolk granules into the extra-embryonic fluid at different stages of development (Figures S1A – S1D, data not shown). Released small blobs fuse to generate larger blobs (Figure S1E). Consistent with a defect in yolk[24], *alfa-1* mutants displayed a decreased survival during starvation-induced L1 diapause (Figure 1E). These results suggest that ALFA-1 functions in the storage or degradation of endocytosed yolk in the embryo.

An HA-tagged ALFA-1 translational reporter was expressed ubiquitously during embryonic development and was enriched in the intestine in later stages of embryonic development. ALFA-1 showed a diffuse cytoplasmic localization similar to that of the human C9orf72 long isoform (the isoform more similar to ALFA-1) in HeLa cells and human motor neurons and embryonic kidney cells under basal cell culture conditions (i. e. in the presence of amino acids)[18,19,25] (Figure 1F). We determined if expression of *alfa-1* or a transgene encoding the long isoform of human *C9orf72* and codon-optimized for expression in *C. elegans* (*ceC9orf72*) expressed under the *alfa-1* promoter could rescue the blob phenotype of *alfa-1(ok3062)* embryos. *alfa-1(+)* transgenes almost completely rescued the blob phenotype, while two *ceC9orf72* transgenes partially rescued this defect (Figure 1G), showing that ALFA-1 and C9orf72 likely function in similar molecular genetic pathways.

We also determined if we could rescue the blob phenotype by expressing *alfa-1* under different promoters driving its expression in the embryonic intestine, neurons, hypoderm or ubiquitously. Only the latter led to a complete rescue of the blob phenotype in *alfa-1* embryos, suggesting that *alfa-1* likely functions cell-autonomously in most embryonic cells to regulate yolk homeostasis (Figure S1B and data not shown).

Genetic interactions between Rab GTPases and *alfa-1* indicate that ALFA-1 functions in a late step of the endolysosomal pathway

To determine at which step in the endolysosomal pathway *alfa-1* functions in yolk storage/degradation, we used RNAi to reduce the functions of putative small Rab GTPases encoded by the *C. elegans* genome[26] and determined how the knockdown of each Rab GTPase affected the penetrance of the blob phenotype in *alfa-1* embryos (Figure 2). We note that when animals were fed the control HT115 bacteria containing the empty vector the penetrance of the blob phenotype was decreased as compared to OP50-fed animals (Figure S2A), likely because the progeny of HT115-fed mothers had less yolk than did the progeny of OP50-fed mothers (Figure S2B).

Knockdown of *rab-7* prevented the blob phenotype of *alfa-1* embryos (Figures 2A, 2B, and 2E). Rab7 is found in late endosomes and marks the early-to-late endosome transition[27]. Knockdown of *rab-7* had been previously reported to cause the accumulation of yolk in enlarged endosomes in wild-type

embryos[28]. Yolk also accumulated in these abnormal endosomes in *alfa-1* embryos, indicating that *alfa-1* functions after *rab-7* and late endosome formation to promote the appropriate storage and/or degradation of yolk (Figure 2B, 2E, top panel).

We observed that knockdown of *rab-6.1*, *rab-8*, *rab-11.1* or *rab-11.2* also suppressed the blob phenotype caused by loss of function of *alfa-1* (Figure 2A). Although we cannot preclude other functions in the storage or degradation of yolk, these Rab GTPases all have been previously implicated in secretion[29–31], suggesting that a decrease in secretion reduces the blob phenotype and consistent with our hypothesis that the blob phenotype is caused by an abnormal release of yolk (Figure 2E, bottom panel). *rab-10* knockdown also suppressed the blob phenotype; however, animals treated with *rab-10* RNAi and their progeny seemed to have less yolk (data not shown) likely reducing the penetrance of the blob phenotype, since less yolk would have been available to be released.

rab-5 and *rab-35* RNAi treatments enhanced the blob phenotype (Figure 2A). RAB-5 regulates early endosome formation after endocytosis, and RAB-35 regulates the endocytic recycling of protein cargo, including of plasma membrane receptors[32,33]. This result suggests the presence of a reuptake mechanism in which RAB-5 is necessary for the endocytosis of the abnormally released yolk in the embryo while RAB-35 is involved in the recycling of the receptor back to the plasma membrane (Figure 2F, top panel). Interestingly,

loss-of-function mutations in the *ALS2* gene, which encodes a Rab5 activator, cause a recessive, juvenile form of ALS[34,35]. That a decreased Rab5 function causes ALS is analogous to our finding that decreased *rab-5* function enhances the *alfa-1* blob phenotype. *rab-1* RNAi enhanced the *alfa-1* blob phenotype (Figure 2A). Recently, C9orf72 has been reported to be a Rab1a effector in the regulation of autophagy[20]. Perhaps *rab-1* regulates yolk metabolism partially through the activity of *alfa-1*.

We also observed an enhancement of the blob phenotype upon *rab-2* RNAi treatment. We further analyzed the relationship between the *alfa-1* blob phenotype and *rab-2* using a deletion allele of *rab-2*, *nu415*, a molecular null allele[36]. *rab-2(nu415)* embryos contained blobs, and *rab-2(nu415); alfa-1(ok3062)* double mutants had blobs larger than those of either single mutant (Figure 2C). These data indicate that RAB-2 is also necessary for normal yolk metabolism and that RAB-2 and ALFA-1 likely work in parallel pathways to regulate yolk storage or degradation (Figure 2F, bottom panel).

smcr-8, the _C. elegans_ ortholog of the C9orf72-interacting protein SMCR8, functions with ALFA-1 to regulate yolk homeostasis

rab-2 adult hermaphrodites are egg-laying defective and accumulate eggs *in utero* with small blobs. *rab-2; alfa-1* hermaphrodites expressing a *vit-2::gfp* reporter accumulate eggs with large blobs visible as GFP-positive puncta in the mother's uterus (Figure 3A). To identify genes that function in the same pathway

as ALFA-1, we performed a genetic screen for enhancers of the *rab-2* blob phenotype (Figure 3A).

The mutation *n5788* both enhanced the *rab-2* blob phenotype and caused blobs by itself (Figures 3C-E), as do *alfa-1(lf)* mutations. *n5788* is a nonsense mutation in the gene *R144.5* (Figure 3B), the *C. elegans* ortholog of human *SMCR8* (Smith-Magenis syndrome chromosome region, candidate 8). We renamed *R144.5* as *smcr-8*. Another nonsense allele of *smcr-8*, *gk173828* (Figure 3B), phenocopied the *n5788* mutation (Figure 3C). The blob phenotype caused by either *smcr-8* mutation was rescued by overexpressing wild-type *smcr-8* (Figure 3C). These results indicate that *n5788* causes a loss of *smcr-8* function and that *smcr-8*, like *alfa-1*, regulates yolk metabolism and enhances the *rab-2* blob phenotype.

alfa-1; smcr-8 embryos did not have an enhanced blob phenotype compared to that of the single mutants (Figures 3D and 3E), and *rab-7* RNAi also suppressed the *smcr-8* blob phenotype (Figure 3F), indicating that *alfa-1* and *smcr-8* act after late endosome formation in the same pathway to regulate endomembrane trafficking events.

In human embryonic kidney cells, C9orf72 levels are greatly reduced upon SMCR8 knock-down[18]. To determine if ALFA-1 levels are decreased in *smcr-8* mutants, we performed western blot analyses of endogenous ALFA-1 in the wild type and in *alfa-1* and *smcr-8* mutant embryos. ALFA-1 protein was not detected in *alfa-1(ok3062)* mutants. The levels of ALFA-1 were decreased in the

absence of SMCR-8, although apparently to a lesser extent than C9orf72 was decreased in human cell lines. We then asked if the decreased levels of ALFA-1 could account for the blob phenotype of *smcr-8* mutants. *alfa-1* overexpression did not rescue the blob phenotype of *alfa-1; smcr-8* embryos, indicating that both *smcr-8* and *alfa-1* are necessary for proper yolk homeostasis (Figures 3G, right panel, and 3H).

Human and mouse SMCR8 can physically interact with C9orf72 to form a complex able to activate Rab39 and Rab8 and promote autophagy[18,19,37]. We tested if *C. elegans* SMCR-8 can physically interact with ALFA-1 and C9orf72 in yeast two-hybrid spot assays. Indeed, SMCR-8 interacted with ALFA-1 and to a lesser extent with C9orf72 (Figure 3I). ALFA-1 and C9orf72 did not interact with a control protein, FLCN-1, a DENN-containing protein like both ALFA-1 and SMCR-8, the human ortholog of which, folliculin, is not known to interact with C9orf72[38]. To validate the physical interaction between ALFA-1 or C9orf72 with SMCR-8 and determine the subcellular localization of this interaction *in vivo*, we used the split GFP (spGFP) system (Figure 3J)[39,40]. ALFA-1 and C9orf72 were tagged with the C-terminal portion of GFP (spGFPC), while SMCR-8 and FLCN-1 were tagged with an N-terminal fragment of GFP (spGFPN). Only upon physical interaction of proteins containing spGFPC and spGFPN can green fluorescence be detected (Figure 3J). We detected interaction when SMCR-8, but not FLCN-1, was combined with ALFA-1 and, to a lesser extent, with C9orf72 (Figure 3K), validating our yeast two-hybrid spot

assay findings. The interactions of ALFA-1 and C9orf72 with SMCR-8 seen in the embryo occurred in the cytoplasm. The decreased interaction between C9orf72 and SMCR-8 compared to that of ALFA-1 with SMCR-8 might explain the partial rescue of the *alfa-1* blob phenotype by the *C9orf72* transgene compared to the *alfa-1* transgene (Figure 1G).

The physical interactions of ALFA-1 and C9orf72 with SMCR-8 indicate that the molecular mechanism of the interaction between *C. elegans* ALFA-1 and SMCR-8 and of that between human C9orf72 and SMCR8 is likely conserved.

Endolysosome homeostasis is altered in *alfa-1* and *smcr-8* mutant embryos

Besides the presence of blobs in *alfa-1* and *smcr-8* mutants, we also noted an abnormal localization of yolk in the heads of mutant embryos. In *alfa-1* and *smcr-8* embryos, yolk granules were localized closer to the lumen of the pharynx than in wild-type embryos (Figures 4A and S3A). This abnormal localization in *alfa-1* and *smcr-8* embryos was rescued by overexpressing wild-type *alfa-1* and *smcr-8*, respectively, confirming that is the loss of function of these genes that causes this abnormal phenotype (Figure 4A).

We determined if any of the compartment(s) of the endolysosomal pathway were similarly mislocalized. Endosomes, ER and Golgi all looked grossly normal in *alfa-1* mutant embryos (Figure S3C). However, the lysosomal markers LMP-1[41] and NUC-1::mCherry[42] showed an abnormal localization similar to that of yolk in *alfa-1* and *smcr-8* mutants (Figures 4A, 4B, S3B). The abnormal

lysosomal localization monitored using the NUC-1::mCherry reporter in *alfa-1* and *smcr-8* embryos was rescued by expressing *alfa-1* or *smcr-8* transgenes, respectively, confirming that *alfa-1* and *smcr-8* are necessary for the normal localization of lysosomes (Figure 4A). We did not observe a decrease in the average intensity of the NUC-1::mCherry reporter in mutant embryos (data not shown), indicating that initial lysosomal biogenesis is not altered in the mutants, in contrast with the recent suggestion of Shi *et al.* [21] based on their studies of ALS patient iPSC-derived motor neurons. The abnormal localization of yolk and lysosomal markers is not caused by the presence of blobs, since suppression of the *alfa-1* blob phenotype by *rab-6* RNAi did not alter the abnormal localization of either yolk or the lysosomal marker NUC-1::mCherry (Figure S4).

To further understand the abnormalities of yolk and lysosomal marker localization in *alfa-1* and *smcr-8* mutants, we determined a colocalization coefficient of the lysosomal marker NUC-1::mCherry with the yolk marker VIT-2::BFP (Figure 4C). We defined yolk granules as VIT-2::BFP signal not colocalized with NUC-1::mCherry, lysosomes as NUC-1::mCherry signal not colocalized with VIT-2::BFP and endolysosomes as the regions where the two fluorescent markers colocalized. The lysosomal marker colocalized significantly more with yolk protein in *alfa-1* mutants than in the wild type (Figure 4C), indicating that the endolysosomal population was increased in the mutant, possibly because of enhanced endolysosome formation and/or impaired lysosome reformation. We observed that although wild-type and *alfa-1* oocytes

endocytosed similar amounts of yolk (Figures S1A and S2B), the average intensity of VIT-2::BFP in both yolk granules and specially endolysosomes was much higher in *alfa-1* embryos than in wild-type embryos, suggesting that *alfa-1* mutants are defective in yolk degradation (Figures 4D). To verify the abnormal degradation of yolk in the mutants, we quantified the intensity of the VIT-2::tdimer2 reporter in the heads of L1 larvae, where yolk mostly localizes in endolysosomes (data not shown). We note that fluorescently-tagged VIT-2 in endolysosomes cannot be detected when using GFP, the fluorescence of which is quenched in the acidic environment of lysosomes. We noticed that *alfa-1* and *smcr-8* L1 animals contained higher levels of the yolk reporter than did wild-type larvae (Figure 4E), consistent with a defect in lysosomal degradation of endocytosed cargo. We also observed defects in the degradation of endocytosed cargo in late larval-stage coelomocytes, macrophage-like cells present in the pseudocoelom (Figure S5A). This finding indicates that defects in the degradation of endocytosed cargo are not limited to the embryo. However, fat degradation was not affected in the intestine of mutant adult animals (Figure S5B).

To further examine the relationship between lysosomal homeostasis and the blob phenotype, we asked if mutations in *glo-1* or *cup-5* either cause blobs or modify the *alfa-1* or *smcr-8* blob phenotype. *glo-1* is a Rab GTPase involved in the biogenesis of lysosome-related gut granules[43], and *cup-5*, the *C. elegans* ortholog of the human mucolipin 1 gene, is required for normal endolysosomal

transport and degradation[44]. We observed that *glo-1(zu391)* mutant embryos did not express the blob phenotype, and only 2% of *cup-5(n3264)* hypomorphic mutant embryos contained blobs (Figure 4F). However, when these mutations were combined with either *alfa-1(ok3062)* or *smcr-8(n5788)*, the blob phenotypes of the *alfa-1* and *smcr-8* mutants were enhanced (Figure 4F). These data support our conclusion that abnormalities in lysosomal homeostasis can lead to the blob phenotype.

We noticed that in wild-type embryos lysosomes formed tubular structures, while in *alfa-1* embryos such structures were less obvious and their lengths were decreased (Figures 4G-I). Lysosomal tubular structures are highly dynamic and appear after macrophage and dendritic cell activation and during lysosome reformation after lysosomal degradation of vesicular cargo[45]. We performed time-lapse microscopy using the lysosomal reporter NUC-1::mCherry and the yolk protein reporter VIT-2::BFP to determine if the tubular structures seen in wild-type embryos were indeed lysosomes in the process of being reformed. We observed that lysosomal tubular structures were derived from endolysosomes – as determined by the presence of both yolk and lysosomal markers – contained little or no VIT-2::BFP reporter, and after formation underwent scission to reform lysosomes (Figure 4H). Tubules in *alfa-1* and *smcr-8* mutants were shorter (Figure 4I) and less abundant (*alfa-1* = 2.1 ± 0.5, *smcr-8* = 2.9 ± 0.6, wild type = 5.8 ± 0.7 tubules/embryo ± s.e.m) than those in wild-type embryos. A decrease of yolk content in the embryo caused by a mutation in the yolk receptor *rme-2*

led to a decrease in tubule length in *rme-2* but not in *alfa-1; rme-2* mutants as compared to wild-type and *alfa-1* embryos, respectively (Figure 4I) and to a suppression of the number of tubules per embryo caused by loss of *alfa-1* (*rme-2* = 7.5 ± 0.8, *alfa-1; rme-2* = 5.3 ± 0.3 tubules/embryo ± s.e.m.; $P > 0.5$). Although this effect of low levels of embryonic yolk on lysosomal tubule length remains to be understood, the suppression of the defect in the number of tubules per embryo caused by *alfa-1* loss of function in the *alfa-1; rme-2* double mutant is consistent with a dependence of lysosomal reformation on there being a low amount of yolk in endolysosomes.

We asked if knock-down of the Arf-like small GTPase *arl-8* would modify the *alfa-1* and *smcr-8* blob phenotypes, since mammalian Arl8 is necessary for lysosomal tubulation in macrophages[46]. Decreased *arl-8* levels led to an enhanced blob phenotype (Figure 4J). We suggest that *arl-8* RNAi treatment enhanced the defect in lysosomal reformation seen in *alfa-1* and *smcr-8* mutants, reducing the number of lysosomes available to degrade yolk and leading to an enhanced abnormal release of yolk in *alfa-1* and *smcr-8* mutants. In short, our data indicate that the defect in yolk degradation likely is responsible for the observed defects in lysosomal reformation in *alfa-1* embryos, since lysosomal degradative function is required for lysosomal reformation[45] and the blob phenotype. However, it remains possible that an increase in degradation of lysosomes is a contributing factor to the decreased lysosomal population compared to the endolysosomal population in the *alfa-1* mutant embryos.

Mutations in the gene that encodes the lysosomal membrane protein *scav-3* decrease the formation of tubular lysosomal structures[47]. However, we did not detect the presence of blobs, the abnormal localization of endolysosomes in the pharynx or a defect in the degradation of yolk in *scav-3(tm3659)* deletion-mutant embryos, suggesting that the mechanism by which *scav-3* mutations lead to defective lysosomal tubulation is different from that of *alfa-1* and *smcr-8* mutations.

Defective lysosomal homeostasis leads to the accumulation of cell corpses and substrates for autophagy

Lysosomes can fuse with late endosomes, phagosomes and autophagosomes to promote the degradation of their cargoes. Phagocytosis and autophagy are essential processes that degrade large foreign particles and cytoplasmic constituents, respectively. We asked if the lysosomal degradation of cargo derived from phagocytosis (apoptotic cell corpses) and autophagy (SQST-1) was defective in *alfa-1* and *smcr-8* embryos.

We determined the number of cell corpses in the heads of 1.5-fold-stage embryos. Compared to wild-type embryos, *alfa-1* and *smcr-8* mutants showed an excess of cell corpses (Figures 5A and 5B). Overexpression of either *alfa-1(+)* or *ceC9orf72* transgenes completely or partially rescued, respectively, the corpse defect of *alfa-1* mutants (Figure 5A), and overexpression of *smcr-8(+)* rescued the *smcr-8* cell-corpse defect (Figure 5B).

Excess cell corpses might be caused by either excessive cell death or a defect in the engulfment or degradation of engulfed cell corpses. To assess the latter, we determined the recruitment of lysosomes to vesicles containing phagocytosed cell corpses[42]. RAB-7-positive cell corpse-containing phagosomes fuse with lysosomes to form phagolysosomes and degrade their content (Figure 5C). *alfa-1* and *smcr-8* embryos showed a decrease in phagolysosome formation (incorporated lysosomes) and an increase in the percentatge of phagosomes with no lysosomes attached (Figure 5D). This defect is consistent with the longer time needed for the incorporation of lysosomes in RAB-7-positive cell corpse-containing phagosomes after RAB-7 clustering around phagosomes in the mutant embryos (Figure 5E), while the average time period for the degradation of cell corpses after incorporation of lysosomes was similar between wild-type and *alfa-1* and *smcr-8* mutant embryos (Figure 5F). These results suggest that the defective degradation of yolk and decreased lysosomal reformation lead to a decrease in available lysosomes to be recruited to apoptotic cell-containing phagosomes, causing a defect in apoptotic-cell clearance.

We asked if autophagic flux also is impaired in *alfa-1* and *smcr-8* embryos. SQST-1, the *C. elegans* homolog of SQSTM1/p62 (sequestosome 1), is selectively removed by autophagy during embryogenesis[48]. To monitor the autophagic flux in wild-type and mutant embryos, we generated an SQST-1::mCherry::GFP dual fluorescent reporter. This reporter exploits the

different sensitivities of GFP and mCherry fluorescence to quenching in low pH environments: the GFP signal, but not the mCherry signal, is quenched by the acidic pH in autophagolysosomes[49]. Hence, this reporter labels autophagosomes and autophagolysosomes as GFP-positive and mCherry-positive particles or as only mCherry-positive particles, respectively. We observed that in both *alfa-1* and *smcr-8* embryos, the percentage of autophagosomes was higher than in wild-type embryos, indicating a defect in autophagolysosome formation in the mutants (Figures 5G and 5H). Mutations in the *C. elegans* orthologs of the autophagy genes *ULK1* and *p62/sequestosome* – *unc-51(e1189)* and *sqst-1(ok2892)*, respectively – did not cause the blob phenotype and knock-down of *let-363*, the *C. elegans* ortholog of the autophagy inhibitor mTOR, did not affect the *alfa-1*, *smcr-8* or *rab-2* blob phenotypes (data not shown), indicating that it is not the impaired autophagy in *alfa-1* and *smcr-8* mutants that leads to the blob phenotype. Using a SQST-1::GFP reporter, we detected an accumulation of the reporter in the heads of *alfa-1* and *smcr-8* adults (Figure S5C), indicating altered autophagy in the mutants. This defective autophagy is consistent with the hypothesis that loss of *C9orf72* function contributes to neurodegeneration -- which might be caused by the accumulation of misfolded and aggregated proteins in C9orf72 ALS patients -- as previously proposed based on studies of mouse and human cell lines [19,37,50].

Our data indicate that *alfa-1* and *smcr-8* are necessary for the maintenance of lysosomal homeostasis and that mutations in either of these genes lead to defective lysosomal degradation of endosomal, phagosomal and autophagosomal cargo.

Discussion

Mutations in *alfa*-1, the *C. elegans* ortholog of the ALS/FTD gene *C9orf72*, lead to motor neuron degeneration and stress sensitivity[8]. Here we describe a novel phenotype of *alfa-1* mutants – the abnormal release of yolk into the extra-embryonic fluid. From our analysis of *alfa-1* we discovered the function of a *C9orf72* homolog: *alfa-1* acts in the degradation of endocytosed material, in lysosomal reformation and in the general maintenance of lysosomal homeostasis (Figures 5I and 5J).

Our data indicate that *C. elegans alfa-1* and human *C9orf72* function in similar genetic molecular pathways, since (1) expression of the long isoform of human *C9orf72* can partially rescue the *alfa-1* mutant phenotype, and (2) from a genetic screen for additional mutations that cause the blob phenotype, we identified *R144.5*, the *C. elegans* ortholog of *SMCR8* (Smith-Magenis syndrome chromosome region 8), a protein that has been reported recently to interact physically with C9orf72 to regulate autophagy[19,37,50].

Our findings about the function of *alfa-1* in the degradation of endocytosed material and the maintenance of lysosomal homeostasis explain apparently disparate previous observations concerning effects of the loss of *C9orf72* function, such as (a) the abnormal accumulation of lysosomes and aggregates of the autophagy target p62/sequestosome seen in *C9orf72* knockout mice, human cells depleted of *C9orf72,* and *C9orf72* ALS/FTD patients[20,37,51–53],

and (b) the defective fusion of phagosomes to lysosomes seen in *C9orf72*-knockout mice[51].

The dominant inheritance of the ALS/FTD caused by a hexanucleotide repeat expansion in *C9orf72* and the lack of *C9orf72*-coding mutations in ALS/FTD patients suggest a gain-of-function pathogenic mechanism for the repeat expansion[3,4]. Nonetheless, some clinical features of ALS/FTD might result from a reduction or loss of C9orf72 function. For example, a recent study showed that haplo-insufficiency of *C9orf72* leads to neurodegeneration of ALS patient iPSC-derived motor neurons in the presence of elevated glutamate levels or upon expression of the Proline-Arginine dipeptide-containing protein associated with C9orf72 ALS[21]. Based on our observations of *alfa-1* mutants, we suggest that *C9orf72* decreased function would similarly cause defects in lysosomal degradation and an abnormal release of vesicular contents, and in that way contribute to the systemic inflammatory response seen in ALS patients[54] and/or to the excessive glutamate release leading to neuronal excitotoxicity caused by overactivation of excitatory glutamate receptors, a process that has been suggested to be a pathogenic mechanism in ALS[55]. In addition, decreased function of *C9orf72* might result in the abnormal degradation and consequent release of pathogenic dipeptide-repeat proteins – the products of the translation of repeat expansion-containing RNAs – promoting their cell-to-cell prion-like spreading and non-autonomous cell toxicity[56] and thereby augmenting the *C9orf72*-driven disease process. Our

observations suggest potential toxic effects of drugs that target *C9orf72* with the goal of ameliorating RNA and/or dipeptide repeat-containing protein pathogenicity.

Acknowledgements

We thank P. Agbu, Z. Zhou, B. Grant, A. Kimura, X. Wang, G. Ou, D. Lee, S. V. Lee, and M. E. Gallegos for providing strains and constructs; R. Droste for determining DNA sequences and RNAi library management; N. An and T. Ljungars for strain management; N. Bhatla, K. Burkhart, D. Denning, A. Doi, T. Hirose, S. Luo and N. Paquin and other members of the Horvitz laboratory for discussions; R. H. Brown, J. Valcárcel and Z. Zhou for suggestions concerning the manuscript; and the *Caenorhabditis* Genetic Center, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440), and the National BioResource project for strains. A. C. was supported by an EMBO postdoctoral long-term fellowship (ALTF 144-2011). An ALS Therapy Alliance grant (2013-S-0113) and gifts from Mr. and Mrs. D. C. Barnard to the M.I.T. McGovern Institute and the Halis Family Foundation to the M.I.T. Aging Brain Initiative helped support this research. H.R.H. is the David H. Koch Professor of Biology at M.I.T. and an Investigator at the Howard Hughes Medical Institute.

Author contributions

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Figure Legends

Figure 1. *alfa-1* mutations cause a defect in yolk storage or transport in the *C. elegans* embryo. (A) Genomic organization of *alfa-1*. Two alleles of *alfa-1*, *gk498021* and *ok3062* are both likely null. *gk498021* is a G-to-A transition leading to a tryptophan-to-opal stop codon at amino acid 54, and *ok3062* is a partial deletion of exons 3 and 4. Black bar, sequence deleted in the *ok3062 mutant*. (B) Differential interference contrast (DIC) and confocal images of wild-type, *alfa-1(ok3062)* and *alfa-1(gk498021)* comma-stage (top panels) and three-fold (bottom panels) embryos containing the yolk protein transgene *pwIs98[vit-2::tdimer2]* or *nIs755[vit-2::bfp, vit-2::pHluorin]*, respectively. Arrowheads, blobs (abnormal yolk presence in extra-embryonic fluid). Scale bars, 10 μm. (C) Confocal images of comma-stage embryos of the indicated genotypes containing the transgene *nIs755[vit-2::bfp, vit-2::pHluorin].* Mutation of *rme-2*, the oocyte's yolk receptor gene, suppresses yolk uptake and the blob phenotype. Scale bar, 10 μm. Animals also contained the reporter *qxIs257[nuc-1::mCherry].* (D) Percent of 3-fold or older embryos of the indicated genotypes containing blobs, showing the suppression of the *alfa-1* blob phenotype caused by loss of the yolk receptor gene *rme-2* function. For the quantification of the penetrances of the blob phenotype, we studied embryos at the 3-fold stage or older but before initiation of pumping and derived from one-day adults unless otherwise stated; Nomarski differential interference contrast (DIC) optics was used. (E) Survival curve of wild-type

(black), *alfa-1(ok3062)* (red) and *alfa-1(gk498021)* (blue) L1 larvae that hatched in the absence of food. The data shown are representative of two independent experiments. (F) anti-HA tag immunofluorescence of proliferative (left) and comma-stage (right) embryos expressing the reporter *nIs759[P_{alfa-1}::alfa-1 gDNA::HA::alfa-1 3'UTR]* showing ALFA-1::HA cytoplasmic localization. Scale bar, 10 μm. (G) Percent of 3-fold or older embryos of the indicated genotypes containing blobs. Overexpression of either wild-type *alfa-1* or codon-optimized *C9orf72* for expression in *C. elegans* (*ceC9orf72*) completely or partially rescued the *alfa-1* blob phenotype, respectively. ‡, animals expressed a *P_{alfa-1}::4xnls::gfp::alfa-1 3'UTR* transcriptional reporter. See also Figure S1.

Figure 2. Knockdown of some Rab GTPases modulates the *alfa-1* blob phenotype and shows ALFA-1 acts late in the endolysosomal pathway. (A) *alfa-1(ok3062)* L1 or L4 (#) animals were treated with the indicated RNAi against *C. elegans* Rab GTPase genes[26] and their progeny scored. The average percent of F1 embryos with blobs from at least three independent experiments is shown. n = 100, except for *rab-1* RNAi where n ≥ 20. Blue bars and red bars correspond to RNAi treatments that enhanced or suppressed the blob phenotype, respectively in a statistically significant manner (Adjusted *P* value < 0.005, One-way ANOVA test). Error bars, s.e.m. (B) DIC and epifluorescene images of comma-stage embryos of the indicated genotypes containing the reporter *pwIs23[vit-2::gfp]* derived from control or *rab-7*-RNAi treated mothers.

rab-7 knockdown caused the accumulation of yolk in enlarged early and late endosomes in wild-type and *alfa-1* embryos and suppressed the *alfa-1* blob phenotype. Scale bar, 10 μm. Arrowheads, blobs. (C) DIC and epifluorescence images of comma-stage (top panels) and late-stage (bottom panels) embryos of the indicated genotypes containing the reporter *vit-2::tdimer2*. *rab-2* loss of function caused the presence of blobs in the extra-embryonic fluid and enhanced *alfa-1* blob phenotype, indicating that *alfa-1* and *rab-2* work in different pathways to regulate yolk homeostasis. (D to F) Proposed genetic pathway that regulates yolk metabolism showing (D) wild-type and *alfa-1* embryos and the (E) suppression and (F) enhancement of the *alfa-1* blob phenotype after reducing the levels of the indicated Rab GTPases. Light coloring indicates low protein activity. See also Figure S2.

Figure 3. *smcr-8* functions in the same pathway as *alfa-1* to regulate yolk metabolism. (A) *rab-2(nu415)* blob phenotype enhancer screen. P_0 *rab-2* animals were mutagenized and the F_2 generation was screened for accumulated F_3 eggs with large blobs detected with a *vit-2::gfp* reporter. m, mutation that enhances *rab-2* blob phenotype. (B) Genomic organization of *smcr-8*. The *n5788* nonsense allele isolated from the genetic screen in A is a C-to-T transition leading to a glutamine-to-ochre stop codon at amino acid 202. The *gk173828* allele is a T-to-A transversion leading to a leucine-to-amber stop codon at amino acid 122. (C) Percent of 3-fold or older embryos of the indicated genotypes

containing blobs*. smcr-8* overexpression rescued the blob phenotype of the two different *smcr-8* nonsense alleles. * Animals contained the *vit-2::gfp* reporter. (D and E) DIC images of embryos containing the (D) *vit-2::tdimer* reporter and (E) average percent and s.e.m. of the progeny with blobs of embryos of the indicated genotypes from three independent experiments. *smcr-8(n5788)* led to presence of blobs and enhanced *rab-2(nu415)* but not *alfa-1(ok3062)* blob phenotypes, indicating that both *alfa-1* and *smcr-8* work in the same genetic pathway and different from that of *rab-2* to regulate yolk homeostasis. Scale bar, 10 μm. In E, embryos were derived from HT115-L4440 (control RNAi)-fed mothers to better observe the blob phenotype enhancement (Figure S2). *, Animals contained the *vit-2::tdimer2* reporter. (F) Average percent of *smcr-8(n5788)* embryos with blobs after treatment with control or *rab-7* RNAi from three independent experiments. s.e.m. is shown. Animals contained the *vit-2::tdimer2* reporter. (G) Western blot analysis of ALFA-1 levels in embryos of the indicated genotypes. A western blot using anti-tubulin antibody was the loading control. Animals carried the *vit-2::tdimer2* reporter. (H) Average percent of embryos of the indicated genotypes with blobs (three independent experiments). s.e.m. is shown. *, Animals carried the *vit-2::tdimer2* reporter. (I) Yeast-two hybrid spot assay showing the interactions of SMCR-8, but not FLCN-1, with ALFA-1 and C9orf72 in plates without histidine (-His) containing the competitive inhibitor of histidine synthesis 3-AT. Serial ¼ dilutions were spotted. (J) Schematic representation of the split-GFP (spGFP) method for

assessing protein-protein interactions *in vivo.* Animals express a C-terminal GFP fragment (spGFPC) fused to protein B in combination with an N-terminal GFP fragment (spGFPN) fused to proteins A or C. Only physical interaction of the spGFPN- and spGFPC-tagged proteins leads to the emission of green fluorescence. (K) DIC and confocal images of late-stage embryos expressing a spGFPC fragment fused to ALFA-1, C9orf72 or by itself (empty) in combination with a spGFPN fragment fused to SMCR-8 or FLCN-1. Physical interaction, monitored by the detection of green fluorescence, was detected only when ALFA-1::spGFPC or C9orf72::spGFPC were combined with SMCR-8::spGFPN, but not FLCN-1::spGFPN. Scale bar, 10 μm.

Figure 4. Yolk degradation is abnormal in *alfa-1* and *smcr-8* mutants. (A, B) DIC and confocal fluorescence images of embryos of the indicated genotypes containing the reporters *vit-2::bfp* and *nuc-1::mCherry* showing the abnormal reporter localization in *alfa-1* and *smcr-8* mutants and the rescue of the abnormal localization by *nEx[alfa-1(+)]* or *nEx[smcr-8(+)]* transgenes, respectively. Red arrowheads, blobs. Scale bar, 10 μm. (B) Confocal images of the endogenous lysosomal protein LMP-1 in comma-stage embryos showing a distribution similar to that of NUC-1::mCherry. LMP-1 was detected by immunofluorescence (IF) using the anti-LMP-1 antibody described in Ref.[57]. Scale bar, 10 μm. Dashed line, embryo outline. (C) Co-localization coefficients of NUC-1::mCherry with VIT-2::BFP (i. e. ratio of NUC-1::mCherry-positive pixels

colocalizing with VIT-2::BFP in endolysosomes to sum of total NUC-1::mCherry-positive pixels) in comma-stage embryos of the indicated genotypes. n ≥ 10. Error bars, s.e.m. **** $P < 0.0001$ (Student's t-test). (D) Quantification of VIT-2::BFP mean fluorescence intensity in comma-stage embryos of the indicated genotypes in yolk granules or endolysosomes. n = 11. Error bars, s.e.m. **** $P < 0.0001$ (Student's t-test). (E) Average intensity of VIT-2::tdimer2 fluorescence in the heads of L1 larvae of the indicated genotypes. Error bars, s.e.m. * $P < 0.05$; ** $P < 0.01$ (Student's t-test). (F) Average percent of embryos of the indicated genotypes with blobs (three independent experiments). Embryos were derived from HT115-L4440-fed mothers. * Animals contained the *nuc-1::mCherry* and the *vit-2::BFP; vit-2::pHluorin* reporters. (G) 1.5-fold embryos of the indicated genotypes carrying the reporter NUC-1::mCherry detected in hypodermal cells. In wild-type embryos, the lysosomal marker accumulates in small aggregates and tubular structures (blue arrow), while in *alfa-1* and *smcr-8* mutants the lysosomal marker accumulates mostly in larger aggregates. Scale bar, 10 μm. Red box, inset. Inset scale bar, 1 μm. (H) Time-lapse confocal microscopy starting at time t showing the formation of a lysosomal tubular structure from an endolysosome and subsequent lysosomal reformation in a wild-type embryo carrying the lysosomal marker NUC-1::mCherry (purple) and the yolk marker VIT-2::BFP (green). Maximum intensity projection along the z-axis of three slices. Arrows indicate reformation tubules. Scale bar, 1 μm. (I) Average length of lysosomal tubules in

embryos of the indicated genotypes. Error bars, s.e.m. ** $P < 0.01$; *** $P < 0.001$ (Student's t-test). n ≥ 5 embryos. (J) Percent of F1 *alfa-1* and *smcr-8* embryos with blobs derived from P0s treated with the indicated RNAi clones. See also Figures S3-S5.

Figure 5. Defective lysosomal degradation leads to the accumulation of cell corpses and substrates for autophagy. (A, B) Average number of cell corpses scored in the heads of (A) *alfa-1* or (B) *smcr-8* 1.5-fold embryos. *nEx[alfa-1(+)]*, *nEx[ceC9orf72]* and *nEx[smcr-8(+)]* indicate overexpression of *alfa-1*, *ceC9orf72* or *smcr-8* rescuing constructs, respectively. n ≥ 13 embryos. Error bars, s.e.m. One-Way ANOVA with Bonferroni *post hoc* test and Student's t-test, for A and B, repectively. ** $P < 0.01$; **** $P < 0.0001$. (C) Time-lapse images of RAB-7::GFP-positive, cell corpse-containing phagosomes with no lysosomes (None), with attaching lysosomes (attaching), and with incorporated lysosomes (Incorporated) that will result in the degradation of the cell corpse (degraded). Scale bar, 1 μm. (D) Percent of cell corpse-containing phagosomes displaying different NUC-1::mCherry localization as classified in **C** for the genotypes indicated. n ≥ 90 corpses, and at least seven 1.5-fold embryos. Error bars, s.e.m. n.s. $P > 0.05$; ** $P < 0.01$; *** $P < 0.001$. (E) Average time from the formation of a RAB-7-positive cell corpse-containing phagosome (t_{None}) until the incorporation of lysosomes ($t_{incorporated}$) leading to the formation of phagolysosomes. n ≥ 13 cell corpses. Error bars, s.e.m. *, $P < 0.05$ (Student's

t-test). (F) Average time from the incorporation of lysosomes ($t_{incorporated}$) until the complete degradation ($t_{degraded}$) of the C1, C2 and C3 cell corpses[58]. n ≥ 5 cell corpses. Error bars, s.e.m. n.s. $P > 0.05$. (G) Confocal fluorescence images of 2-fold embryos of the genotypes indicated containing the reporter *nIs845[sqst-1::mCherry::gfp]* showing defective fusion of lysosomes with autophagosomes in *alfa-1* and *smcr-8* embryos. (H) Average percent of autophagosomes (GFP- and mCherry-positive particles) of all SQST-1-containing particles (i.e., autophagosomes and autophagolysosomes). n = 5 embryos. Error bars, s.e.m. *, $P < 0.05$ (One-way ANOVA). (I) In wild-type animals, lysosomes fuse with endosomes, phagosomes and autophagosomes to generate endolysosomes, phagolysosomes and autolysosomes, respectively, leading to the degradation of their contents. After degradation, lysosomes are reformed. (J) In *alfa-1* or *smcr-8* embryos, lysosomes fuse with endosomes but both degradation of yolk and lysosome reformation are impaired, leading to the abnormal release of yolk to the extra-embryonic fluid. Defects in the formation of phagolysosomes and degradation of autophagosomal content are also observed.

STAR METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, H. Robert Horvitz (horvitz@mit.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

C. elegans hermaphrodite strains were maintained on Nematode Growth Medium (NGM) plates containing 3 g/L NaCl, 2.5 g/L peptone and 17 g/L agar supplemented with 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KPO_4 and 5 mg/L Cholesterol with *E. coli* OP50 as a source of food[59]. All strains were derived from the Bristol N2 wild-type strain and are listed in Table S2. All strains were maintained at 20°C. RNAi treatments were performed by feeding animals with HT115 bacteria containing different RNAi constructs[60–62]. Unless otherwise stated, 25-30 L1 larvae were placed onto each NGM plate containing 1 mM isopropyl-β-D-thiogalactoside and 100 μg/ml and about 72 hours later the progeny of these animals were scored for presence of blobs. Control RNAi treatments were performed using the empty vector pL4440. The names of the RNAi clones used in this study are provided in the Table S2.

METHOD DETAILS

Plasmids

The P_{alfa-1}*::4xnls::gfp* transcriptional reporter was constructed using PCR to amplify a 2.5 Kb fragment upstream of *alfa-1* into 25 nucleotides in exon 2, to include the two alternative first exons in *alfa-1*. The resulting amplicon was digested with XbaI and SmaI and ligated into pPD122.56, which encodes a 4xNLS::GFP fusion to generate P_{alfa-1}*::4xnls::gfp::unc-54 3'UTR*. *unc-54 3'UTR* was replaced by the 1.0 Kb region downstream of the stop codon of *alfa-1*. The P_{alfa-1}*::alfa-1 gDNA::3xGly::HA::alfa-1 3' UTR* was generated using site-directed mutagenesis of the plasmid P_{alfa-1}*::alfa-1 gDNA::alfa-1 3' UTR*, which contains 2.5 Kb 5' of *alfa-1*, the full-length *alfa-1* gDNA and 1.0 Kb 3' of the stop codon of *alfa-1*. P_{alfa-1}*::ceC9orf72 cDNA::3xGly::HA::alfa-1 3' UTR* was generated from P_{alfa-1}*::alfa-1 gDNA::3xGly::HA::alfa-1 3' UTR* by blunt ligation of a wild-type human *C9orf72* cDNA codon-optimized for expression in *C. elegans* containing three synthetic introns generated using gBlocks® Gene Fragments (Integrated DNA Technologies) after the eighth amino acid of *alfa-1* exon 2 and eliminating the downstream *alfa-1* ORF. The *ceC9orf72* sequence is shown as supplementary information. The *vit-2* reporters were cloned in the pRS426 plasmid and contain 1 Kb of the promoter and the full-length *vit-2* gDNA, followed by a linker of five glycines and the specified fluorescent protein (mCherry, BFP or pHluorin) and the *unc-54* 3'UTR. For the split-GFP protein-protein *in vivo* interaction assay, all cloning was performed using the Clontech In-Fusion® system. The C-terminal GFP (spGFPC) fragment was cloned between *alfa-1* or *C9orf72* and the *alfa-1* 3'UTR. To generate the empty

spGFPC transgene, spGFPC was cloned between the *alfa-1* promoter and the 3'UTR. The *smcr-8::spGFPN* transgene was generated by first cloning an *smcr-8* PCR product encompassing a region from 1.2 Kb upstream to 250 bp downstream of *smcr-8* ORF using a Zero Blunt™ TOPO™ PCR Cloning Kit and then adding the spGFPN fragment between smcr-8 ORF and 3'UTR. Once this plasmid was generated, to construct the *flcn-1::spGFPN* transgene we substituted the *smcr-8* ORF with a *flcn-1a* cDNA. The *sqst-1::mCherry::gfp* reporter was cloned in the pRS426 plasmid and contained 2.5 Kb of the promoter and the full-length *sqst-1* gDNA[48], followed by mCherry and GFP fluorescent genes, with a linker of three glycines between each ORF, followed by the *unc-54* 3'UTR. The P_{elt-2}*::alfa-1::unc-54 3'UTR* transgene was cloned by replacing the *alfa-1* promoter in a P_{alfa-1}*::alfa-1 gDNA::unc-54 3' UTR* transgene derived from P_{alfa-1}*::4xnls::gfp* with the promoter of the *elt-2* gene[63]. The specific primer sequences are described in Key Resources Table and Table S1.

Germline transformation

Germline transformation was performed by injecting the specified amounts of the indicated plasmids into the gonads of one-day adults. F1 animals containing the extrachromosomal array were singled into individual plates and those transmitting the array to their progeny were maintained to generate independent transgenic lines[64]. *alfa-1* and *ceC9orf72* translational reporters were injected at 50 µg/ml into *alfa-1(ok3062); lin-15(n765)* animals with 20 µg/ml of the

indicated fluorescent markers and 40 µg/ml of the *lin-15(n765)*-rescuing plasmid pL15EK as co-injection markers[65]. A *smcr-8* PCR product encompassing 1.2 Kb upstream to 250 bp downstream of *smcr-8* ORF was injected in *smcr-8(n5788)* and *smcr-8(gk173828)* animals at 50 µg/ml with 20 µg/ml of the ubiquitously expressed P_{sur-5}*::nls::gfp* marker. Vitellogenin fluorescent reporters were injected at 50 µg/ml into *lin-15(n765)* or *alfa-1(ok3062); lin-15(n765)* animals with 40 µg/ml of pL15EK as a co-injection marker. The *nls755* transgene was generated by injection of 50 µg/ml of *vit-2::BFP* and 50 µg/ml of *vit-2::pHluorin* transgenes into *alfa-1(ok3062); wjIs51* animals. Extrachrosomal arrays were integrated by gamma-ray irradiation (4,800 rads) of transgene-carrying L4 animals. For the split-GFP protein-protein *in vivo* interaction assay, wild-type animals were injected with 50 µg/ml of the spGFPN-containing transgene, 50 µg/ml of the spGFPC-containing transgene and 5 µg/ml *Pges-1::mCherry*, used as co-injection marker.

Starvation survival assay

Synchronized L1 larvae were incubated in 2.5 mL of sterilized M9 buffer on a rocker at 20°C for the times indicated. Approximately 100 worms were placed on individual seeded plates at the indicated times. The number of survivors was determined after 3 days at 20°C. Day 1 is considered to be the first day of starvation and was used as the 100% survival point.

Microscopy, immunohistochemistry and image analysis

Nomarski DIC and epifluorescence micrographs were obtained using an Axioskop II (Zeiss) compound microscope and OpenLab software (Agilent). Confocal microscopy was performed using Zeiss LSM 510, LSM 710 and LSM 800 instruments. The resulting images were prepared using ImageJ software (National Institutes of Health). Image acquisition settings were calibrated to minimize the number of saturated pixels and were kept constant throughout each experiment. Graphs and indicated statistical analyses were performed using Microsoft Excel and GraphPad Prism 6 and 7 softwares. For immunohistochemistry, fixing the samples were fixed for 5 min at -20°C in methanol followed by incubation at -20°C in acetone. Samples were then rehydrated through 70%, 50% and 30% ice-cold acetone in PBS[66]. anti-HA primary antibody (ab9110, Abcam) incubation was overnight at 4°C. Secondary antibody was goat anti-rabbit antibody coupled with Alexa 488 (1:2500) (Invitrogen). Images were acquired using a Zeiss LSM 510 confocal microscope. To determine lysosomal tubulation and reformation (Figure 4H), we took Z-stack images using Zeiss LSM 800 confocal microscope at 0.37 μm z-intervals every ≈14 seconds. To quantify lysosomal tubule length (Figure 4I), we photographed the hypoderm of comma-stage embryos carrying the *nuc-1::mCherry* reporter, and the lengths of tubular structures were quantified using ImageJ. To determine the average time for phagolysosome formation or cell-corpse degradation (Figures 5E and 5F), we took Z-stack images using Zeiss LSM 800

confocal microscope at 0.5 μm z-intervals every 2 minutes. To analyze the % of autophagosomes from the total of autophagosomes and autophagolysosomes (Figures 5G and 5H), we acquired images of the hypoderm of 2-fold embryos using a Zeiss LSM 800 confocal microscope and determined the number of particles positive for both GFP and mCherry (autophagosomes) and the number of particles positive for only mCherry (autophagolysosomes) uisng ImageJ. To detect the release and merging of blobs (Figure S1C), we took Z-stack images using Zeiss LSM 710 or LSM 800 confocal microscopes at 0.3-0.5 μm z-intervals approximately every 70 sec. To rule out the possibility of previously released blobs appearing from a focal plane not imaged, we excluded blobs initially visible in the first or last focal planes to ensure that we could detect their release. To detect the fusion of blobs (Figure S1E), we took Z-stack images using Zeiss LSM 700 confocal microscope at 0.37 μm z-intervals approximately every 70 seconds.

Counts of blobs and persistent corpses

The penetrance of the blob phenotype, i.e. the percent of animals in which one or more blobs were visible, was quantified in embryos at the 3-fold stage or older but before initiation of pumping, derived from one-day adults unless otherwise stated. We used a ×100 objective equipped with Nomarski differential interference contrast (DIC) optics. The number of persistent cell corpses was

scored by counting raised highly refractile dead cells in the head region of 1.5-fold stage embryos[58].

Genetic screen and mapping of *smcr-8(n5788)*

smcr-8(n5788) was isolated from a genetic screen of *rab-2(nu415); pwIs23 [vit-2::gfp]* animals for mutations that cause the F3 embryos that accumulate in the uteri of F2 progeny to contain large GFP-positive blobs. Mutagenesis was performed by treating synchronized L4 larvae with 0.05 M of ethyl methanesulfonate (EMS) at 20 °C for 4 hours. *smcr-8(n5788)* was identified by crossing the mutant isolate with a Hawaiian strain containing the *nIs755* reporter, isolating the F2 progeny with accumulated eggs with large blobs and mapping the mutation using single nucleotide polymorphisms[67] and whole-genome sequencing.

Antibody production and western blot analysis

A protein fragment corresponding to amino acids 124-190 of ALFA-1 fused to glutathione S- transferase (GST) was expressed, purified using glutathione Sepharose 4B (Amersham Biosciences) and used to raise rabbit anti-ALFA-1 antibodies. Antisera were generated by Pocono Rabbit Farm and Laboratory. Specific antibodies were affinity-purified using an identical ALFA-1 protein fragment fused to maltose-binding protein (MBP) and coupled to Affigel 10 (Bio-Rad). Protein extracts were prepared from *C. elegans* embryos of the

indicated genotypes containing the yolk reporter *pwIs98[vit-2::tdimer2]*. 10 µg of total protein were loaded onto Any kD™ Mini-PROTEAN® TGX™ precast protein gels and then transferred to nitrocellulose membranes. The membranes were probed with anti-ALFA-1 and mouse anti-tubulin (Sigma) antibodies. Immunocomplexes were detected using HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibodies (Invitrogen) followed by chemiluminescence (Western Lightning ECL, PerkinElmer).

Yeast two-hybrid binding assay

alfa-1, *C9orf72*, *smcr-8* and *flcn-1a* cDNAs were cloned into pGBKT7 and pGADT7 plasmids, and the constructs were sequentially introduced into yeast strain PJ649A[68]. 1mL of exponentially growing yeasts were pelleted and resuspended in 70 µl of water. 10 ul of 10 ug/mL denatured salmon sperm, 1 µg of the desired DNA, 240 µl 50% PEG 3350 and 36 µl 1 M LiOAc were added to the resuspended yeasts. After 30' at room temperature, the mix was heat shocked for 15' at 42°C. Yeasts were washed with water and plated on the appropriate SD plates[69]. Single colonies were streaked and cultured for two days at 30°C on SD plates containing minimal supplements without tryptophan and leucine. To test yeast growth, yeast strains were grown overnight in liquid, and cultures were diluted to a similar optical density at 600 nm. Serial 4x dilutions were made in water, and 5 µl of each dilution was used to yield one spot. Plates were cultured for two days at 30°C on SD plates containing minimal

supplements without tryptophan, leucine and histidine and containing 3 mM of the competitive inhibitor of histidine synthesis 3-AT.

Oil Red O (ORO) staining and imaging

Well-fed or food-deprived one-day adult worms were fixed by washing them in 60% isopropanol, stained overnight with 0.3% ORO in 60% isopropanol and washed with 1xPBS with 0.01% Triton[70]. Worms were imaged using a Nikon DS-Ri2 color camera coupled to a Nikon SMZ18 microscope.

***ceC9orf72* sequence**

The long isoform of C9orf72 was codon-optimized for expression in *C. elegans* and synthesized using gBlocks® Gene Fragments (Integrated DNA Technologies). Three synthetic introns (in italics and lower case) were introduced to improve *ceC9orf72* expression.

ATGTCAACCCTCTGTCCACCACCGAGTCCAGCAGTTGCGAAAACTGAGATTG
CTCTCTCTGGAAAATCCCCTCTCCTGGCCGCTACTTTCGCGTACTGGGATAAT
ATTCTGGGACCACGAGTGCGTCATATTTGGGCCCCGAAAACAGAACAGGTAC
TCCTTAGCGATGGTGAAATCACGTTCCTGGCAAATCACACGTTGAACGGAGAA
ATCCTTAGAAACGCGGAGTCAGGAGCCATTGATGTAAAATTTTTCGTTCTCTCG
GAGAAG*gtaagtttaaacatatatatactaactaaccctgattatttaaattttcag*GGTGTTATTATCG
TTAGCCTGATTTTTGACGGTAACTGGAACGGTGATCGTAGTACTTATGGCCTTA

GTATTATTCTGCCACAAACTGAACTCTCTTTCTATCTTCCACTCCATCGAGTTTG

CGTAGATCGATTGACACATATAATAAGAAAAGGAAGAATTTGGATGCATAAAGA

GCGTCAGGAGAATGTTCAAAAGATTATTCTTGAAGGAACTGAACGAATGGAAG

ACCAAGGACAATCAATTATCCCAATGCTGACAGGCGAGGTGATTCCAGTTATG

GAACTCTTATCATCGATGAAGTCGCACTCCGTTCCAGAGGAAATTGATATCGC

CGATACGGTATTAAACGACGATGATATTGGCGATTCTTGCCACGAGGGATTCC

TTCTTAATGCAATCTCCTCACACTTGCAAACTTGTGGTTGTTCAGTGGTTGTCG

GATCTTCTGCCGAAAAG*gtaagtttaaacagttcggtactaactaaccatacatatttaaattttcag*G

TGAATAAAATTGTCCGAACTCTGTGTCTCTTCTTGACCCCAGCAGAACGTAAAT

GTTCTCGACTGTGTGAGGCCGAGTCTTCTTTCAAATATGAAAGTGGATTGTTCG

TGCAAGGACTTTTGAAAGATTCAACTGGATCTTTTGTCCTCCCATTCCGACAAG

TGATGTATGCCCCATACCCTACTACACACATCGATGTTGATGTAAATACGGTGA

AGCAAATGCCACCGTGCCATGAGCACATCTATAATCAGCGACGTTACATGAGA

AGCGAGCTTACAGCATTCTGGCGAGCTACATCCGAAGAAGATATGGCCCAAG

ACACTATTATTTACACCGACGAGAGCTTTACGCCTGACCTGAACATATTTCAGG

ATGTTCTTCATCGAGACACCCTGGTCAAAGCTTTTCTTGATCAAGTTTTCCAATT

AAAACCAGGTCTGTCCCTCAGATCCACATTCTTAGCCCAATTTCTGCTTGTCTT

ACATCGTAAAGCTCTGACTTTGATTAAGTACATTGAG*gtaagtttaaacatgattttactaac*

*taactaatctgatttaaattttcag*GATGATACGCAAAAGGGAAAAAAACCTTTCAAATCAC

TTAGAAATCTCAAAATTGACCTCGATCTGACTGCAGAAGGAGATTTAAACATAA

TTATGGCCTTAGCAGAAAAGATCAAGCCAGGTTTACACTCTTTCATATTCGGAA

GACCGTTTTATACAAGTGTTCAGGAGAGAGACGTGTTAATGACTTTT

QUANTIFICATION AND STATISTICAL ANALYSIS

Unless otherwise stated, for quantification of the blob phenotype penetrance, 100 embryos at the 3-fold stage or older but before initiation of pumping derived from one-day adults were scored per genotype or RNAi treatment. The average value and the standard deviation of the mean (s.e.m) of three independent experiments are shown. Non-parametric Student's t-tests were used to determine statistical significance. For Figure 2A, multiple comparison one-way ANOVA was used to determine statistical significance. Graphs and indicated statistical analyses were performed using Microsoft Excel and GraphPad Prism 6 and 7 softwares. For colocalization coefficient quantification and VIT-2::BFP mean intensity quantification (Figures 4C and 4D), a region of interest (ROI) was drawn around each embryo, avoiding blobs and cell corpses. Another ROI was drawn inside the embryo where no reporter was visible to set the background threshold for each image. Analysis was performed with Zen software (Zeiss).

Table S1. Additional Oligonucleotides. Related to STAR Methods.

Table S2. *C. elegans, E. coli* and *S. cerevisiae* Strains. Related to STAR Methods.