The amino acid transporter SLC-36.1 cooperates with PtdIns3P 5-kinase to control phagocytic lysosome reformation

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Phagocytic removal of apoptotic cells involves formation, maturation, and digestion of cell corpse–containing phagosomes. The retrieval of lysosomal components following phagolysosomal digestion of cell corpses remains poorly understood. Here we reveal that the amino acid transporter SLC-36.1 is essential for lysosome reformation during cell corpse clearance in Caenorhabditis elegans embryos. Loss of slc-36.1 leads to formation of phagolysosomal vacuoles arising from cell corpse–containing phagosomes. In the absence of slc-36.1, phagosome maturation is not affected, but the retrieval of lysosomal components is inhibited. Moreover, loss of PPK-3, the C. elegans homologue of the PtdIns3P 5-kinase PIKfyve, similarly causes accumulation of phagolysosomal vacuoles that are defective in phagocytic lysosome reformation. SLC-36.1 and PPK-3 function in the same genetic pathway, and they directly interact with one another. In addition, loss of slc-36.1 and ppk-3 causes strong defects in autophagic lysosome reformation in adult animals. Our findings thus suggest that the PPK-3–SLC-36.1 axis plays a central role in both phagocytic and autophagic lysosome formation.

Introduction

Lysosomes are major sites of cellular degradation, signal sensing, and signal transduction (Luzio et al., 2007; Settembre et al., 2013; Xu and Ren, 2015; Perera and Zoncu, 2016; Davidson and Vander Heiden, 2017). Dysfunction of lysosomes contributes to many human disorders including lysosome storage diseases and neurodegenerative disorders (Saftig and Klumperman, 2009; Ferguson, 2015). Lysosomes receive and degrade both intracellular and extracellular cargoes that are generated by autophagy, endocytosis, and phagocytosis (Luzio et al., 2007). These degradation activities quickly consume the pool of lysosomes in the cell. Thus, lysosomes need to regenerate following lysosomal degradation so as to maintain the homeostasis of the lysosome pool. To meet the demands of cellular degradation, the number of lysosomes can also be increased by activation of TFEB and TFE3, two transcription factors of lysosomal and autophagy genes (Settembre et al., 2011; Martina et al., 2014). TFEB and TFE3 promote transcription of lysosomal and autophagy genes by cytoplasm-to-nucleus translocation in mTOR-dependent or -independent manners (Li et al., 2016; Puertollano et al., 2018).

Recent studies have shed light on the mechanisms underlying lysosome reformation accompanying lysosomal degradation of autophagic and endocytic cargos. Lysosome reformation from autolysosomes, also referred to as autophagic lysosome reformation (ALR), involves phosphatidylinositol 4,5-bisphosphate- and clathrin-mediated membrane budding on autolysosomes, KIF5B-driven elongation of membrane tubules along microtubules, dynamin 2–dependent proto-lysosome scission, and finally proto-lysosome maturation (Chen and Yu, 2017, 2018). Spinster, a lysosomal sugar transporter, was also found to be essential for ALR in cells with prolonged starvation (Rong et al., 2011). Endocytic lysosome reformation is an ATP-dependent process, which also requires lysosomal acidification and intralysosomal Ca2+ (Pryor et al., 2000). In addition, the phosphatidylinositol 3-phosphate (PtdIns3P) 5-kinase PIKfyve and the lysosomal calcium channel TRPML1 are required for endocytic lysosome reformation (Nicot, 2006; Miller et al., 2015; Bissig et al., 2017). PIKfyve generates phosphatidylinositol 3,5-bisphosphate, which activates TRPML1 to control lysosomal Ca2+.

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efflux (Dong et al., 2010; McCartney et al., 2014). Notably, PIKfyve, TRPML1, and mTOR were shown to regulate phagosome and entolic vacuole shrinkage (Krajcovic et al., 2013; Krishna et al., 2016), suggesting that these factors are important for lysosome regeneration on phagolysosomes. Nevertheless, the mechanisms underlying phagocytic lysosome reformation (PLR) remain mostly elusive.

Caenorhabditis elegans provides excellent model for studying phagocytic clearance of apoptotic cells. In the lifetime of a C. elegans hermaphrodite, 131 somatic cells and about half the germ cells undergo apoptosis that is essentially controlled by a linear genetic pathway (Wang and Yang, 2016). The resulting cell corpses are recognized and phagocytosed by neighboring cells (Sulston and Horvitz, 1977; Sulston et al., 1983; Gumienny et al., 1999; Conradt et al., 2016). Two major signaling pathways, ced-1/6/7 and ced-2/5/12, act redundantly to recognize and transduce engulfment signals to induce cytoskeleton reorganization of the engulfing cell, leading to cell corpse internalization and formation of membrane-bound phagosomes (Reddien and Horvitz, 2004). The cell corpse–enclosing phagosomes then undergo progressive acidification by sequentially interacting with early and late endosomes, and finally fuse with lysosomes to yield phagolysosomes (Flannagan et al., 2012; Wang and Yang, 2016). This process, termed phagosome maturation, involves multiple factors that are shared by endocytic trafficking and autophagy (Kinchen and Ravichandran, 2008). For example, small Rab GTPases and their effectors and regulators, including RAB-5, VPS-34, TBC-2, RAB-14, RAB-2, RAB-7, ARL-8, and the HOPS complex, act cooperatively to regulate phagosome maturation (Li et al., 2009; Xiao et al., 2009; Guo et al., 2010; Sasaki et al., 2013). Within the phagolysosomes, cell corpses are digested by lysosomal acidic hydrolases (Xu et al., 2014). However, an important question that remains unanswered is how lysosomes reorganize following phagolysosomal digestion of cell corpses.

In this study, we characterized PLR in the process of cell corpse clearance during C. elegans embryonic development. We reveal that SLC-36.1, which is homologous to the mammalian neutral amino acid transporters SLC36A1–4 (PAT1–4), functions as an essential regulator of PLR. We further demonstrate that PPK-3, the C. elegans PIKfyve homologue, is required for PLR and that SLC-36.1 and PPK-3 act together to promote PLR during embryonic development. In addition, we show that the SLC-36.1–PPK-3 axis is required for lysosome reformation from autophagosomes in adult animals. Thus, SLC-36.1 and PPK-3 not only are essential for PLR during embryonic cell corpse clearance but also serve as critical regulators in ALR in adult animals.

Results

Loss of slc-36.1 leads to formation of embryonic cell corpse–derived vacuoles

To identify new factors that participate in phagocytic removal of apoptotic cells in C. elegans, we performed an ethyl methanesulfonate (EMS) mutagenesis screen to look for mutants that exhibit altered morphologies or numbers of embryonic cell corpses. By screening ∼12,000 haploid genomes, we identified a group of mutants that contained a number of vacuolar structures. These embryonic vacuoles, which are distinct from the conventional button-like apoptotic cell corpses, were rarely seen in WT (N2; Fig. 1A). Seven of these mutants, yq51, 80, 84, 90, 93, and 95, and II0, had comparable numbers of embryonic vacuoles and failed to complement one another (Fig. 1B), indicating that they affected the same gene. We mapped these mutations to linkage group (LG) III and identified mutations in the Y43F4B.7 gene, which encodes a putative membrane protein that shares sequence homology to the mammalian lysosomal neutral amino acid transporters SLC36A1–4 (Fig. 1C; Sagné et al., 2001; Agulhon et al., 2003). Y43F4B.7 was thus renamed slc-36.1. The slc-36.1 mutants yq51, 80, and 81 contained single nucleotide mutations that cause point mutations in the encoded protein, and the yq80, 90, 95, and II0 mutants had mutations in exon–intron splicing sites, leading to mis-splicing of the pre-mRNA and consequently to frame shifting of the ORF. Because the yq110 mutation caused an early stop codon in the slc-36.1 gene, it should be a strong loss-of-function allele of slc-36.1. Transgenic expression of WT slc-36.1 driven by its own promoter strongly reduced the number of vacuoles in slc-36.1(yq110) embryos (Fig. 1D), confirming that loss of slc-36.1 was responsible for formation of the embryonic vacuoles.

To determine the identities of the embryonic vacuoles, we traced the development of WT and slc-36.1(yq110) embryos. In WT (N2) embryos, the button-like apoptotic cell corpses progressively shrank and disappeared within 30 min (27.6 ± 2.4 min; Fig. 1E and F). In slc-36.1(yq110) embryos, while ~18% of the button-like cell corpses condensed and disappeared as in WT, the majority of cell corpses did not shrink but instead exhibited a morphological switch from button-like to vacuoles (Fig. 1E and F), indicating that these vacuoles were derived from apoptotic cell corpses. To consolidate this conclusion, we constructed double mutants of slc-36.1(yq110) with egl-1(n3082), ced-3(n77), and ced-4(n162), the strong loss-of-function mutations of the essential apoptosis genes egl-1, ced-3, and ced-4 (Ellis and Horvitz, 1986; Conradt and Horvitz, 1998; Gumienny et al., 1999; Horvitz, 2003). Barely embryonic vacuoles were observed in these double mutants (Fig. 1G). Thus, the vacuoles in slc-36.1(yq110) mutant embryos resulted from apoptotic cell corpses.

SLC-36.1 is a putative amino acid transporter that localizes to lysosomes

SLC-36.1 and its mammalian homologues SLC36A1–4 have similar topologies with 11 transmembrane domains (Fig. 2A). C. elegans SLC-36.1, mammalian SLC36A1–4 and LYAAT-1, Drosophila melanogaster Path, and yeast VTA4 are members of the amino acid/auxin permease superfamily; they all contain a conserved iGSGi motif essential for the functions of amino acid transporters (Fig. 2B; Broe, 2014). Except for yeast VTA4, the other proteins are H+-coupled amino acid transporters and contain a conserved histidine residue responsible for binding and translocating the protons required for transporter activity (Metzner et al., 2008). To determine if the amino acid transporter activity of SLC-36.1 is involved in formation of embryonic vacuoles, we expressed SLC-36.1 proteins with mutations in either the conserved histidine or the iGSGi motif. Compared with
the WT SLC-36.1 that fully rescued the vacuole phenotype in slc-36.1(yq110) embryos, mutant SLC-36.1 carrying mutations of the conserved residues (H57A, G66A, and L68S) or a deletion of iGsGif failed to completely rescue the abnormal embryonic vacuoles in slc-36.1(yq110) (Fig. 2 C). No rescuing activity was observed in slc-36.1(yq110) mutants expressing SLC-36.1 with a P130L mutation identified in slc-36.1(yq84) (Fig. 2 C). In contrast, mutation of Ser 69, which is not conserved in other transporters (Fig. 2 B), did not affect SLC-36.1 rescuing activity (Fig. 2 C). Thus, the amino acid transporter activity is important for slc-36.1 in embryonic
**Figure 2. SLC-36.1 is a putative lysosomal amino acid transporter.** (A) Topology models of *C. elegans* SLC-36.1 and *H. sapiens* SLC36A1. (B) Alignment of the amino acid transporter domains of *C. elegans* SLC-36.1, human SLC36A1-4, rat LYAAT-1, fly Path, and yeast AVT4. Identical residues are shaded in black, and similar ones in gray. The conserved H57, G66, and L68 are indicated in orange, yellow, and green, respectively. The blue star indicates the unconserved Ser 69. The mutation site in slc-36(yq84) mutants (P130L) is indicated in pink. (C) Quantification (mean ± SEM) of embryonic vacuoles in N2 and slc-36.1(yq110) and slc-36.1(yq110) mutants carrying the indicated transgenes. 15 embryos at the 1.5-fold stage were scored for each strain. Three independent transgenic lines were analyzed for each transgene. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Black asterisks indicate comparisons between nontransgene and each SLC-36.1 transgenic line. Red asterisks indicate comparisons of transgenes between WT and mutant SLC-36.1 transgenic lines. (D) Quantification (mean ± SEM) of embryo lethality in N2, slc-36.1 and laat-1 single mutants and laat-1;slc-36.1 double mutants without or with supplementation of amino acids. 250 or more embryos were
development, we examined the hatching ratio of slc-36.1(yq110) embryos and found that it was similar to WT (Fig. 2 D). Surprisingly, double mutants of slc-36.1(yq110) with laat-1(qx42) (Liu et al., 2012) or two additional laat-1 mutants identified in this study, yq20 and yq81, showed 100% embryonic lethality (Fig. 2 D). Consistent with this, double mutants of slc-36.1(yq110) with the laat-1(yq295) weak loss-of-function mutant showed 54% embryonic lethality (Fig. 2 D). Supplementing the culture medium with glycine significantly ameliorated the embryonic lethality of laat-1(yq295);slc-36.1(yq110) double mutants, comparable to the rescuing effect by adding both lysine and arginine, the LAAT-1 substrates (Fig. 2 D). Given that LAAT-1 exports lysine and arginine out of the lysosome and its loss did not obviously affect embryonic survival (Liu et al., 2012), these findings suggest that slc-36.1 likely acts as a neutral amino acid transporter that is required for embryonic development by coordinating with other lysosomal transporters to export amino acids from lysosomes.

We next investigated the expression and subcellular localization of SLC-36.1. Using the CRISPR/Cas9 assay, we knocked in the GFP-coding sequence right after the ATG initiation codon in the slc-36.1 gene. Endogenous levels of GFP::SLC-36.1 were observed in embryos and multiple adult tissues, including the pharynx, intestine, hypodermis, and germ line, and the signal was enriched on the plasma membrane (Fig. S1 A). In the macrophage-like coelomocytes, GFP::SLC-36.1 localized to the membranes of lysosomes positive for the lysosomal DNase II NUC-1 (NUC-1::mCherry [mCh]; Fig. S1 B). Because the signal from GFP::SLC-36.1 was relatively weak, we further generated an mCh-fused SLC-36.1 (mCh::SLC-36.1) driven by the promoter of the engulfment cell-specific gene ced-1. In embryos, mCh::SLC-36.1 was found to localize to the plasma membrane and phagosomes that were positive for the lysosomal lysine/arginine transporter LAAT-1 fused with GFP (LAAT-1::GFP; Fig. 2 E). In the hypodermis, mCh::SLC-36.1 colocalized well with LAAT-1::GFP; Fig. 2 E). In addition, mCh::SLC-36.1 with H57A, G66A, or L68S mutations that reduced the rescuing activities of SLC-36.1 (Fig. 2 C) also colocalized with LAAT-1::GFP to phagosomes and lysosomes (Fig. 2 F). Altogether, these findings suggest that SLC-36.1 localizes and functions on lysosomes.

**slc-36.1 acts at a late stage of phagocytic cell corpse clearance**

Phagocytic removal of cell corpses requires their engulfment followed by delivery to lysosomes (Wang and Yang, 2016). To understand how SLC-36.1 plays a role in this process, we first examined whether cell corpse engulfment was defective in slc-36.1(yq110) embryos. In *C. elegans*, the ced-1/6/7 and ced-2/5/12 signaling pathways act redundantly to control cell corpse engulfment; thus, cell corpse engulfment is mostly blocked in ced-1(e1735);ced-5(n1812) double mutants (Reddien and Horvitz, 2004). In ced-1(e1735);slc-36.1(yq110);ced-5(n1812) triple mutants, the number of conventional button-like cell corpses remained similar to that in ced-1(e1735);ced-5(n1812) double mutants (Fig. 3 A); however, the number of embryonic vacuoles was greatly reduced compared with slc-36.1(yq110) single mutants (Fig. 3 B). This suggests that slc-36.1 likely acts downstream of the engulfment genes. Consistent with this, the recruitment and release of the engulfment receptor CED-1::GFP to cell corpses in slc-36.1(yq110) was similar to that in WT (Fig. 3 C). However, the vacuoles in slc-36.1(yq110) embryos were negative for CED-1::GFP (Fig. 3 D and Fig. 4 F). Together, these data suggest that slc-36.1 functions after CED-1 release from cell corpse-containing phagosomes (Chen et al., 2010).

We next investigated whether SLC-36.1 acts in phagosome maturation. We introduced into slc-36.1(yq110) mutants transgenic arrays expressing fluorescently tagged phagosome maturation factors, including YFP::2x FYVE, an indicator of phagosomal PtdIns3P; GFP::RAB-7, a small GTPase required for late endosome–phagosome fusion; LAAT-1::GFP, a lysosomal lysine/arginine transporter required for phagosome digestion; NUC-1::mCh, a lysosomal DNase; and CPL-1::mCh::ONT, a lysosomal cathepsin protease (Kinchen and Ravichandran, 2008; Guo et al., 2010; Liu et al., 2012; Xu et al., 2014). The button-like cell corpses in slc-36.1(yq110) embryos were positive for these phagosome maturation factors at a similar level to WT (Fig. 4, A–E), indicating that loss of slc-36.1 did not affect phagosome maturation. However, the vacuoles were highly enriched for lysosomal components, including LAAT-1::GFP, NUC-1::mCh, and CPL-1::mCh::ONT, but not phagosomal factors that act at earlier stages (Fig. 4, A–D and F). Thus, SLC-36.1 does not regulate phagosome maturation for phagolysosome formation, and the vacuoles in slc-36.1 embryos were phagolysosomes. To consolidate this conclusion, we used high-voltage EM (HVEM) to examine the ultra-structures of cell corpses and vacuoles in both WT and slc-36.1 embryos. WT cell corpses were more densely stained than living cells and could be classified into several groups, including unengulfed corpses (8.6%), corpses engulfed in early phagosomes (32.8%), and degrading phagolysosomes (50.0%; Fig. 4, G and H). Unlike the densely stained WT cell corpses, 66.3% of cell corpses in slc-36.1(yq110) embryos appeared as electron-lucent, and had few contents (Fig. 4, G and H). This suggests that phagolysosomes form in slc-36.1(yq110) embryos and have degradation capacities but fail to shrink. Using HIS-24::GFP, an apoptotic chromatin marker, and LAAT-1::mCh to monitor the dynamics of phagolysosomes, we found that HIS-24::GFP signals similarly disappeared following phagosomal recruitment of LAAT-1::mCh in both WT and slc-36.1(yq110) embryos (Fig. S2 A), suggesting that phagolysosomes are acidic in

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slc-36.1(yq110) mutants. In addition, slc-36.1(yq110) mutants, like WT, displayed normal lysosomal degradation of caveolin (GFP::CAV-1; Sato et al., 2006; Audhya et al., 2007), the low-density lipoprotein receptor RME-2 (RME-2::GFP; Grant and Hirsh, 1999), and the yolk protein VIT-2 (VIT-2::GFP; Grant and Hirsh, 1999) in early stage embryos. In contrast, these proteins accumulated in laat-1(qx42) loss-of-function mutants (Liu et al., 2012; Fig. S2, B–D). Altogether, these findings suggest that loss of slc-36.1 does not affect lysosomal degradation but leads to formation of phagolysosomal vacuoles.

**slc-36.1 is required for lysosome reformation from phagolysosomes**

To understand how phagolysosomal vacuoles formed in slc-36.1 embryos, we simultaneously monitored the dynamics of HIS-24::mCh and LAAT-1::GFP. In WT embryos, HIS-24::mCh-positive cell corpses first recruited LAAT-1::GFP-positive lysosomes, which then progressively shrank and finally reached sizes similar to general lysosomes (Fig. 5, A and E; and Video 1). A more detailed time-lapse analysis indicated that, accompanying the shrinkage, phagolysosomes generated and released LAAT-1-positive tubules, forming new lysosomes that could be distinguished from the preexisting lysosomes (Fig. S3). This suggests that degradation of phagosomal contents occurred concomitantly with the retrieval of lysosomal components from the phagolysosome. In slc-36.1(yq110) embryos, HIS-24::mCh-positive phagosomes similarly recruited LAAT-1::GFP-marked lysosomes, confirming that phagolysosome formation was not defective (Fig. 5, A and C). However, the size of the phagolysosomes remained similar during the whole monitoring period (1 h), and barely any LAAT-1-positive tubules were generated on the phagolysosomes (Fig. 5, A, D and E; and Video 2). Thus, loss of slc-36.1 does not affect phagosomal recruitment and fusion with lysosomes, but inhibits the retrieval of lysosomal contents from phagolysosomes. To test this further, we colabeled phagolysosomes with LAAT-1::GFP and NUC-1::mCh and performed time-lapse imaging. In WT, subsequent to incorporation of NUC-1::mCh into LAAT-1::GFP-positive phagolysosomes, both LAAT-1::GFP- and NUC-1::mCh-positive tubules were observed to bud from phagolysosomes concomitantly with the reduction of phagolysosome size (Fig. 5, B and E; and Video 3). In slc-36.1(yq110) mutants, however, barely any...
Figure 4. *slc-36.1* functions after phagosome maturation. (A–D) Images of cell corpse labeling by the phagosomal markers YFP::2xFYVE (A), GFP::RAB-7 (B), LAAT-1::GFP (C), and CPL-1::mChImt (D) in N2 and *slc-36.1*(yq110) embryos. Arrows and arrowheads indicate button-like cell corpses and vacuoles, respectively. Boxed regions are magnified (2×) in insets. Bars, 2 µm. (E) Quantification (mean ± SEM) of button-like cell corpses positive for phagosomal markers in N2 and *slc-36.1*(yq110) embryos. 15 embryos were scored at the 1.5-fold stage for each strain. Error bars represent SEM. (F) Quantification (mean ± SEM) of embryonic vacuoles positive for phagosomal markers in *slc-36.1*(yq110) embryos. 15 embryos were scored at the 1.5-fold stage for each strain. (G) HVEM images of a living cell and apoptotic cell corpses at the indicated phagosomal stages in N2 embryos (upper panels) and a phagolysosomal vacuole in a *slc-36.1*(yq110) embryo (lower panels). M, Mitochondria. Bars, 0.2 µm. (H) Quantification of cell corpses shown in G. The numbers of cell corpses analyzed for each genotype are indicated on the top of the columns.
such tubules were observed to form on phagolysosomes, while fusion of lysosomes with phagolysosomes still occurred (Fig. 5 B and Video 4). Together, these findings suggest that slc-36.1 is required for reformation of lysosomes from phagolysosomes.

**PPK-3/PIKfyve is required for PLR**

Our genetic screen also identified yq24 mutants that exhibited embryonic vacuoles similar to slc-36.1 mutants (Fig. 6 A). Double mutants of yq24 with ced-4(n1162) showed neither button-like

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**Figure 5.** slc-36.1 is essential for PLR. (A) Time-lapse chasing of LAAT-1::GFP– and HIS-24::mCh–positive phagolysosomes in N2 and slc-36.1(yq110) embryos. The time point that the LAAT-1::GFP ring was first detected on a cell corpse was set as 0 min. Arrows and arrowheads indicate lysosome fusion and lysosome tubulation, respectively. Bars, 2 μm. (B) Time-lapse chasing of LAAT-1::GFP– and NUC-1::mCh–positive phagolysosomes in N2 and slc-36.1(yq110) embryos. The time point that the LAAT-1::GFP ring was first detected on a cell corpse was set as 0 min. Arrows and arrowheads indicate lysosome fusion and lysosome tubulation, respectively. Bars, 2 μm. (C–E) Quantification (mean ± SEM) of lysosome fusion (C), tubulation (D), and change in phagolysosome sizes (E). 15 cell corpses were analyzed. **, P < 0.01; ***, P < 0.001.
Figure 6. **PPK-3/PKfyve is required for PLR.** (A) Images of LAAT-1::GFP– and HIS-24::mCh–positive embryonic cell corpses and vacuoles in N2, pppk-3(yq24), and pppk-3(n2668) comma-stage embryos. Arrows and arrowheads indicate button-like cell corpses and vacuoles, respectively. Boxed regions are magnified (2.5×) in insets. Bars, 2 µm. (B) Time-course quantification of embryonic vacuoles in the indicated strains. 15 embryos were scored at each stage for each strain. ***, P < 0.001. (C) Time-lapse chasing of a LAAT-1::GFP– and HIS-24::mCh–positive cell corpse in a pppk-3(n2668) embryo. The time point that the LAAT-1::GFP ring was first detected on the cell corpse was set as 0 min. The arrow indicates lysosome fusion. Bars, 2 µm. (D and E) Quantification (mean ± SEM) of lysosome tubulation (D) and change in phagolysosome sizes (E). 15 cell corpses were analyzed. **, P < 0.01; ***, P < 0.001. (F) DIC image of an N2 comma-stage embryo showing the cell corpse-engulfing ABplaapppp cell and fluorescent images of N2, slc-36.1(yq110), and pppk-3(n2668) comma-stage embryos expressing...
apoptotic cell corpses nor vacuolar structures (Fig. 6 B). The yq24 embryonic vacuoles were enriched for both LAAT-1::GFP and HIS-24::mCh (Fig. 6 A). These data suggest that yq24 embryonic vacuoles were phagolysosomes arising from apoptosis. Further genetic analysis revealed that yq24 mutants carried an SI448L mutation in PPK-3, the C. elegans homologue of mammalian PtdIns3P 5-kinase PIKfyve. We thus examined ppk-3(n2668) strong loss-of-function mutants (Nicot, 2006). ppk-3(n2668) embryos contained many vacuolar structures of different sizes, a subset of which were positive for both LAAT-1::GFP and HIS-24::mCh (Fig. 6 A), indicating that they were phagolysosomes. Using time-lapse imaging, we found that HIS-24::mCh–positive cell corpses recruited LAAT-1::GFP–positive lysosomes to form phagolysosomes in ppk-3(n2668) embryos, similar to WT (Fig. 5 A, Fig. 6 C, and Video 5). However, no tubules were observed to form on the phagolysosomes, which failed to condense and remained similar in size during the whole monitoring period (Fig. 6, C–E). In addition, we observed that LAAT-1::GFP–positive lysosomes kept being recruited to the phagolysosomes (Fig. 6 C). These findings suggest that PPK-3, like SLC-36.1, is required for lysosome retrieval/reformation from phagolysosomes. In addition, we examined the amount of lysosomes in the cell corpse-engulfing ABplaapppp cells (Li et al., 2013). LAAT-1::GFP–positive lysosomes were significantly reduced in ABplaapppp cells compared with those in WT (Fig. 6, F and G), suggesting PLR is important for maintenance of the regular number of lysosomes within engulfing cells.

**SLC-36.1 acts together with PPK-3**

We next investigated whether SLC-36.1 and PPK-3 act together to control PLR. ppk-3(n2668) embryos had a higher number of HIS-24::mCh–positive vacuoles than slc-36.1(yq110) embryos; however, double mutants of slc-36.1(yq110) with ppk-3(n2668) exhibited a similar number of HIS-24::mCh–positive vacuoles to ppk-3(n2668) single mutants (Fig. 7, A and B). Using time-lapse imaging, we monitored phagolysosome condensation and lysosomal reformation in slc-36.1(yq110);ppk-3(n2668) double mutants. Phagolysosomes in slc-36.1(yq110);ppk-3(n2668) embryos showed similar kinetics of size reduction to ppk-3(n2668) single mutants (Fig. 7 C). Consistent with this, the lysosome reformation defect was not further enhanced in slc-36.1(yq110);ppk-3(n2668) double mutants compared with ppk-3(n2668) single mutants (Fig. 7 D). These data suggest that slc-36.1 and ppk-3 function in the same genetic pathway to control lysosome reformation.

We next examined the localization of PPK-3 and SLC-36.1 and found that GFP::PPK-3 and mCh::SLC-36.1 colocalized to phagosomes (Fig. 7 E). Using the microscale thermophoresis (MST) assay, we further found that EGFP::SLC-36.1 and Myc–His–PPK-3 form a complex (Fig. 7 F). To investigate if they interact directly, we generated and purified GST–SLC-36.1(1–53) and GST–SLC-36.1(101–142), which contain the cytosolic N terminus and loop 2 of SLC-36.1, respectively (Fig. 7 G). In GST pull-down assays, GST–SLC-36.1(101–142) directly interacted with 35S-labeled PPK-3(1–668) and PPK-3(669–1497) (Fig. 7 G), suggesting that SLC-36.1 and PPK-3 interact at multiple sites. Altogether, these findings suggest that SLC-36.1 acts together with PPK-3 to control PLR.

**The SLC-36.1–PPK-3 axis is essential for ALR**

Finally, we investigated whether the SLC-36.1–PPK-3 axis also plays an essential role in ALR in adult animals, in which no cell death occurs. Interestingly, slc-36.1(yq110) adult animals displayed enlarged vacuolar structures that were positive for LAAT-1::GFP, NUC-1::mCh, and GFP::LGG-2 in the hypodermis, which were rarely seen in the WT (Fig. 8 A and Fig. 54). This suggests that the enlarged vacuoles in slc-36.1(yq110) hypodermis were likely autolysosomes. The autophagosome formation defective atg-2(bp576) mutants (Lu et al., 2011) showed no obvious enlargement of LAAT-1::GFP– and NUC-1::mCh–lysosomes (Fig. 8 A). In double mutants of slc-36.1(yq110) with atg-2(bp576), the enlarged LAAT-1::GFP– and NUC-1::mCh–positive vacuoles were restored to atg-2(bp576) levels (Fig. 8, A and E). Importantly, starvation greatly increased the sizes of hypodermal vacuoles in slc-36.1(yq110), but this change was suppressed by atg-2(bp576) (Fig. 8, B and E). RNAi of lgg-1 or lgg-2, the C. elegans homologues of Atg8/LC3, similarly suppressed starvation-induced enlargement of NUC-1::mCh–positive lysosomes in slc-36.1(yq110) hypodermis (Fig. 8 C). Together, these findings suggest that loss of slc-36.1 caused accumulation of enlarged autolysosomes in the hypodermis. Like slc-36.1 mutants, ppk-3(yq24) mutants displayed enlargement of LAAT-1::GFP– and NUC-1::mCh–positive vacuoles in the hypodermis following starvation treatment, and this change was suppressed by atg-2(bp576) or RNAI of lgg-1 or lgg-2 (Fig. 8, D–F). With normal feeding, the ppk-3(n2668) strong loss-of-function mutants accumulated numerous enlarged NUC-1–positive autolysosomes, similar to starvation-treated ppk-3(yq24) mutants (Fig. 8, D, F, and G). Importantly, the double mutants of slc-36.1(yq110) with ppk-3(n2668) contained enlarged autolysosomes similar to ppk-3(n2668) single mutants (Fig. 8, G and H), suggesting that slc-36.1 acts with ppk-3 to regulate autolysosomes. To determine if slc-36.1 and ppk-3 are required for ALR, we compared the dynamics of NUC-1::mCh lysosomes in hypodermal cells in WT with NUC-1::mCh vacuoles in slc-36.1 and ppk-3 mutant hypodermal cells. In the WT, NUC-1::mCh tubules frequently extended from lysosomes (Fig. 8 I and Video 6). In contrast, no tubules were found to extend from the enlarged autolysosomes in slc-36.1(yq110) and ppk-3(n2668) mutants (Fig. 8 I and Videos 7 and 8). These data suggest that slc-36.1 and ppk-3 are essential for ALR. To consolidate this point, we examined lysosomes in the hypodermis using HVEM. In WT, the majority of lysosomes (84.6%) are electron dense with an average area of 0.48 μm² (Fig. 9, A and D). Remarkably, around 10% of lysosomes were observed to extend electron-lucent tubules (Fig. 9, B).
Figure 7. **SLC-36.1 functions together with PPK-3 in PLR.** (A) Images of LAAT-1::GFP– and HIS-24::mCh–positive cell corpses and phagolysosomal vacuoles in slc-36.1(yq110) and ppk-3(n2668) single mutants and slc-36.1(yq110);ppk-3(n2668) double mutants. Arrows and arrowheads indicate cell corpses and phagolysosomal vacuoles, respectively. Boxed regions are magnified (2.5×) in insets. Bars, 2 µm. (B) Quantification (mean ± SEM) of the sizes of HIS-24::mCh–positive vacuoles. 15 embryos were scored at the comma stage for each strain. ***, P < 0.001. (C and D) Quantification (mean ± SEM) of the change in phagolysosome sizes (C) and lysosome tubulation events (D). 15 cell corpses were quantified for each strain. ***, P < 0.001. (E) Colocalization of GFP::PPK-3 and mCh::SLC-36.1 to phagosomes in an N2 embryo. Boxed regions are magnified (2.5×) in insets. Bars, 2 µm. ***, P < 0.001. (F) Binding curve of Myc–His–PPK-3 to EGFP::SLC-36.1 measured by MST. EGFP was used as the negative control. ΔFNorm, normalized fluorescence ratio of initial fluorescence to fluorescence after laser heating. (G) Purified GST–SLC-36.1(1-53) or GST–SLC-36.1(1-142) was incubated with 35S-labeled PPK-3(1-668 or 669-1497) and pulled down with glutathione-Sepharose beads. Precipitated proteins were resolved by SDS-PAGE and viewed with autoradiography.
Figure 8. The SLC-36.1–PPK-3 axis is required for ALR. (A and B) Images of LAAT-1::GFP– and NUC-1::mCh–positive vacuoles in the posterior hypodermis in adult animals of the indicated genotypes without (A) or with starvation (B). Bars, 5 µm. (C) Images of NUC-1::mCh–positive hypodermal vacuoles in N2 and slc-36.1(yq110) adults treated with control (Ctrl) or lgg-1 or lgg-2 RNAi under normal and starvation conditions. Bars, 5 µm. (D) Images of LAAT-1::GFP– and
and E). In general, formation of these tubules appeared to involve polarization, tubulation, extension, and scission (Fig. 9 B). These electron-lucent lysosomal tubules are consistent with those observed with fluorescent microscopy and scission (Fig. 9 I). In slc-36.1(yq110) and ppp-3(yq24) mutant hypodermis, however, the average area of lysosomes was greatly increased (average area of 0.89 and 0.69 µm², respectively; Fig. 9, C and D). Moreover, 43.4% and 49.4% of these enlarged lysosomes were electron-lucent, and lysosomal tubules were barely observed (Fig. 9 E). In ppp-3(n2668) strong loss-of-function mutants, hypodermal lysosomes were even larger than in ppp-3(yq24) and slc-36.1(yq110) mutants, which were mostly electron-lucent with no lysosomal tubules (Fig. 9, C–E). Altogether, these findings further suggest that the SLC-36.1–PPK-3 axis is required for ALR in adult animals.

Discussions

Although the regulators and mechanisms controlling cell corpse engulfment and phagosome maturation have been mostly revealed, little is known about the retrieval of lysosomal contents, i.e., lysosome reformation, the terminal step of cell corpse clearance. In this study, we characterized PLR during C. elegans embryonic development. We revealed that the lysosomal amino acid transporter SLC-36.1 and the PtdIns3P 5-kinase PPK-3 are essential regulators of lysosome reformation following phagolysosomal digestion of cell corpses. Our results indicated that loss of slc-36.1 did not impair cell corpse engulfment and the subsequent phagosome maturation process; instead, it strongly delayed the reformation of lysosomes from phagolysosomes. Failure of lysosome reformation blocked the shrinkage of cell corpse-degrading phagolysosomes, resulting in formation of vacuoles in embryos. At the same time, our genetic screen also identified ppp-3 mutants that show similar embryonic vacuoles to slc-36.1 mutants. We further found that loss of ppp-3 function, like slc-36.1, abrogated lysosome reformation from phagolysosomes. Double mutants of slc-36.1 with ppp-3 did not show an enhanced embryonic vacuole phenotype compared with single ppp-3 strong loss-of-function mutants, which suggests that slc-36.1 and ppp-3 function in the same genetic pathway. In addition, SLC-36.1 and PPK-3 colocalize to phagosomes, and they physically interact with one another. These facts suggest that SLC-36.1 and PPK-3 act together to control PLR in the process of apoptotic cell removal during embryonic development in C. elegans.

Our findings revealed that, in adult hypodermis where no cell death occurs, SLC-36.1 and PPK-3 play an essential role in ALR. Under normal feeding conditions, slc-36.1 strong loss-of-function mutants show enlarged lysosomes in the hypodermis. With starvation, the abnormal lysosomal enlargement was greatly enhanced. The enlargement of lysosomes in normally fed or starved animals was inhibited by loss of atg-2 and RNAi of lgg-1 or lgg-2, indicating that the enlarged lysosomes are autolysosomes generated by autophagy. Using live-cell imaging and HVEM, we found that no lysosomal tubulation occurred on the enlarged autolysosomes in slc-36.1 and ppp-3 hypodermis, which contrasts with the frequent budding and tubulation of lysosomes in WT. Our genetic data suggest that, like embryonic PLR, adult ALR requires the SLC-36.1–PPK-3 axis. In addition, given that PPK-3 was found to regulate terminal lysosome maturation (Bissig et al., 2017), it is likely that the SLC-36.1–PPK-3 axis also plays a pivotal role in lysosome reformation from endolysosomes.

In mammalian cells, the PtdIns3P 5-kinase PIKfyve regulates vacuole shrinkage and cargo redistribution during engulfment, which is achieved in part through the PIKfyve effector TRPML1 (Nicot, 2006; Krishna et al., 2016; Bissig et al., 2017). PIKfyve promotes the export of amino acids and other nutrients from vacuoles concomitantly with vacuole shrinkage to support the growth of engulfing cells during starvation (Krishna et al., 2016). In addition, TRPML1 was found to be required for the scission of lysosomes during lysosome reformation from endolysosomes (Miller et al., 2015). However, neither PIKfyve nor TRPML1 is a lysosomal nutrient transporter that executes the function of nutrient export from vacuoles/lysosomes. Our study now establishes SLC-36.1 as an essential regulator of phagocytic and ALR, which suggests that SLC-36.1 is likely responsible for export of amino acids, e.g., neutral amino acids, from phagolysosomes and autolysosomes. Failure to export amino acids likely prevents lysosome reformation from phagolysosomes and autolysosomes. Remarkably, export of amino acids from phagolysosomes and autolysosomes is essential for the development of embryos, as evidenced by the fact that double mutants of slc-36.1 with laat-1 display high penetrance of embryonic lethality, though slc-36.1 and laat-1 single mutant embryos develop fairly well (Fig. 2 D). These findings suggest that the function of PPK-3/PIKfyve in the regulation of cargo redistribution is likely achieved through amino acid transporters. Because mutations affecting SLC-36.1 transporter activity strongly reduced its rescuing effect on the defective phagolysosome shrinkage (Fig. 2 D), it is likely that amino acid pumping is coupled to PIKfyve-dependent lysosomal tubulation, which ensures that lysosomal shrinkage coincides with export of amino acids. It remains to be determined whether PPK-3/PIKfyve acts through phosphatidylinositol 3,5-bisphosphate to promote the activities of SLC-36.1 and other amino acid transporters, such as LAAT-1. In addition, given that PIKfyve and PATI and PAT4, two mammalian homologues of SLC-36.1, have been found to be important for mTOR activation (Heublein et al., 2010; Zheng et al., 2016; Shang et al., 2017), it is reasonable to hypothesize that the...
Figure 9. **HVEM examination of lysosomes in the hypodermis of N2, slc-36.1(yq110), and ppk-3(yq24) adult animals.** Membrane-bound spherical organelles filled with membrane debris were identified as lysosomes. (A) HVEM images of electron-dense and electron-lucent lysosomes in the hypodermis of N2 animals. Bars, 0.5 µm. (B) HVEM images (top) of lysosomes undergoing different steps of lysosomal reformation in N2 hypodermis. Traces of lysosome reformation at each step are shown underneath. Bars, 0.5 µm. (C) HVEM images of electron-lucent lysosomes in the hypodermis of slc-36.1(yq110), ppk-3(yq24), and ppk-3(n2668) animals. Bars, 0.5 µm. (D) Quantification (mean ± SEM) of lysosome sizes in N2, slc-36.1(yq110), ppk-3(yq24), and ppk-3(n2668) adult hypodermis. 50 lysosomes were measured for each genotype. *, P < 0.05; **, P < 0.01; ***, P < 0.001. (E) Percentage of lysosomes with different morphologies shown in A–C in N2, slc-36.1(yq110), ppk-3(yq24), and ppk-3(n2668) adult hypodermis. For each genotype, indicated number of lysosomes from ≥10 electron microscopic sections of two or three worms were analyzed. (F) Graphic summary of the SLC-36.1–PPK-3 axis in lysosome reformation from phagolysosomes and autolysosomes.

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SLC-36.1 regulates phagocytic lysosome reformation

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SLC-36.1–PPK-3/PKfyve axis plays a pivotal role in mTOR activation in the process of phagocytic clearance of apoptotic cell corpses as well as autophagy, similar to the requirement for the sugar transporter spinster (Rong et al., 2011). PPK-3/PKfyve may also activate SLC-36.1 through physical interaction, as is the case for PKfyve regulation of several membrane transporters, such as the Ca²⁺ channel TRPV6 (Sopjani et al., 2010), the glucose cotransporter SLC5A1 (Shojaiefard et al., 2007), the neutral amino acid transporter SLC6A19 (Bogatikov et al., 2012), and the glutamate transporters EAAT2, EAAT3, and EAAT4 (Gehring et al., 2009; Klaus et al., 2009; Alesutan et al., 2010). Our findings also suggest that, in addition to SLC-36.1, other amino acid transporters (e.g., LAAT-1/PQLC2), may be regulated by PKfyve to promote lysosomal export of degradation products, which facilitates lysosome reformation from phagolysosomes and autolysosomes as well as endolysosomes.

**Materials and methods**

**C. elegans strains**

The N2 Bristol strain was used as the WT. slc-36.1 mutants (yq51, 80, 84, 90, 93, 95, and 110), laat-1 mutants (yq20, 81, and 295), and ppp-3(yq24) mutants were isolated by EMS mutagenesis. Other mutant alleles used in this study are listed by LGs: LG I ced-1(e1735); LG II, laat-1(px42); LG III, ced-4(n162); LG IV, ced-3(n177), ced-5(n182); LG V, egl-1(n3082); and LG X, ppp-3(n2668). The integrated arrays bsl1 (P*spa3::VIT-2::GFP), pwlsl16 (P*spa3::RME-2::GFP), and pwls281 (P*pie::GFP::CAV-1) were provided by B. Grant (Rutgers University, Piscataway, NJ). The integrated array bplsl220 (P*spa3::GFP::LGG-2) was provided by H. Zhang (Institute of Biophysics, Chinese Academy of Sciences, Beijing, China). opis334 (P*ced-1::YFP::2xFYVE) was provided by K.S. Ravichandran (University of Virginia, Charlottesville, VA). The integrated arrays msls18 (HIS-24::GFP) and msls34 (P*ced-1::GFP) were provided by D. Xue (University of Colorado, Boulder, CO). The integrated array yqis135 (P*pie-H2B::mCh + P*nhr-2::HIS-24::mCh) was provided by Z. Du (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China). Other strains used in this study carrying integrated or extrachromosomal arrays were generated in the authors’ laboratories and are as follows: yqlsl66 (P*cicl-30::GFP::SLC-36.1), yqIsl84 (P*cicl-30::mCh::SLC-36.1), yqsl91 (P*cicl-30::GFP::PPK-3), qxlsl66 (P*cicl-30::GFP::RAB-7), qxlsl297 (P*cicl-30::UNC-1::mCh), qxlsl353 (P*cicl-30::LAAT-1::mCh), qxlsl354 (P*cicl-30::LAAT-1::GFP), qxlsl468 (P*mypo::LAAT-1::GFP), qxyex106, 1107, 1108 (P*cicl-30::SLC-36.1), qxyex1109, 1110, 1113 (P*cicl-30::SLC-36.1::ΔΔGesGil), qxyex1165, 1166, 1167 (P*cicl-30::SLC-36.1::H57A), qxyex1168, 1169, 1170 (P*cicl-30::SLC-36.1::P130LL), qxyex1171, 1172, 1173 (P*cicl-30::SLC-36.1::G66A), qxyex1174, 1175, 1176 (P*cicl-30::SLC-36.1::L68S), qxyex1177, 1178, 1179 (P*cicl-30::SLC-36.1::S69A), qxyex1188, 1189, 1190 (P*cicl-30::mCh::SLC-36.1::H57A), qxyex1191, 1192, 1193 (P*cicl-30::mCh::SLC-36.1::G66A)), and qxyex1194, 1195, 1196 (P*cicl-30::mCh::SLC-36.1::L68S). The WormBase IDs of mutants and transgenic arrays are summarized in Table S1. The expressing vectors are listed in Table S2. Animals carrying the integrated arrays were outcrossed with the N2 strain four times. Deletion strains were outcrossed with the N2 strain at least four times. C. elegans cultures and genetic crosses were performed according to standard procedures.

**Mutant isolation and cloning of slc-36.1, pppk-3, and laat-1**

Synchronized L4-stage N2 animals were treated with 50 mM EMS for 4 h. The F2 embryos of EMS-treated animals were observed under differential interference contrast (DIC) microscopes. yq24, 51, 80, 84, 90, 93, 95, and 110, and 295 mutants were isolated based on the presence of embryonic vacuoles. yq51 was mapped to the right end of LG III at the genetic map position +20.67(snp-WBVar00247063) by single nucleotide polymorphism mapping. Whole genome sequencing revealed a C to T mutation in exon 11 of Y43F4B.7, which results in substitution of Ser 336 with Phe. yq80, 84, 90, 93, and 110 failed to complement with yq51, and mutations in Y43F4B.7 were detected in these mutants. yq84 and yq93 mutants contained mutations in exons 4 and 12 of Y43F4B.7, resulting in substitutions of Pro 336 with Leu and Gly 394 with Glu, respectively. yq110 mutants contained a point mutation (GA to AA) in the splicing acceptor site after exon 3, which leads to deletion of exon 3 (89 bp) in the transcript, resulting in an early stop codon in the encoded protein. yq90, 95, and 80 mutants contained point mutations (AG to AA) in the splicing acceptor sites before exons 7, 10, and 12. These mutations led to deletion of exon 7 (98 bp), the first 32 bp of exon 10, and exon 12 (107 bp), respectively, resulting in early stop codons. Transformation rescuing experiments were performed by injecting a plasmid containing the Y43F4B.7 ORF. All mutants were backcrossed with N2 animals at least four times.

yq24 mutants failed to complement pppk-3(n2668) mutants. The yq24 mutation caused a SI448L mutation in the PPK-3 protein. yq20, 81, and 295 mutants did not complement laat-1(qx42). The yq20, 81, and 295 mutations caused P266L, G270R, and C274Y, respectively, in the LAAT-1 protein.

**Predication of topology and transmembrane regions**

Topology models were exported from the Protter website. Transmembrane regions were predicted with the Phobius algorithm.

**Generation of knock-in worms using CRISPR/Cas9**

The insertion of tags at specific sites by CRISPR/Cas9 was performed as described before (Paix et al., 2014). Briefly, a single guide RNA (sgRNA) target site (‘-TCTCTGATAGCCGCTTCTCT GCAGG-3’) in the slc-36.1 gene was used. The specific sgRNA target sequence was introduced into the vector pPD162-Pgst-3 CAS9-Pgst::sgRNA that expresses the Cas9 enzyme and sgRNA. GFP coding sequence with a 33-bp homologous arm containing four synonymous point mutations to prevent recutting was used as the homology-directed repair template. dpy-10 was used as the positive selection marker as reported previously (Paix et al., 2014). Dumpy or roller F1 worms were examined for recombinant by PCR. The recombinants were sequenced to confirm if homology-directed repair occurred correctly. The primers used are listed in Table S3.

**Quantification of embryonic cell corpses and vacuoles**

Embryos mounted on 2% agar pads were anesthetized in 3 µl M9 buffer (1 liter contains 3 g KH₂PO₄, 6 g Na₂HPO₄, 5 g NaCl, and 1 mM MgSO₄) containing 33 mM sodium azide. The numbers of button-like cell corpses and pit-like vacuoles in the head region are presented in Table S2.
of living embryos at various developmental stages (comma, 1.5-fold, 2-fold, 2.5-fold, 3-fold, and 4-fold) were scored using DIC optics. 15 embryos at each developmental stage were scored for each strain. To examine embryonic cell corpse duration, embryos at the two-cell stage were mounted on 2% agar pads in egg salt buffer (118 mM NaCl and 48 mM KCl) at 20°C. Images in 30 Z-sections (1.0 µm/section) were captured every minute for 400 min using an Axiomager M2 microscope (ZEISS) equipped with an AxioCam monochrome digital camera (ZEISS). Images were processed and viewed using ZEN 2 pro software (ZEISS).

Quantification of phagosomal markers
WT and slc-36.1(yq110) embryos carrying CED-1::GFP, YFP::2xFYVE, GFP::RAB-7, LAAT-1::GFP, NUC-1::mCh, or CPL-1::mChOnt transgene were mounted on 2% agar pads in egg salt buffer. Images of 1.5-fold stage embryos in 25 Z-sections (1.0 µm/section) were captured using an Axiomager M2 microscope (ZEISS). The percentage of button-like cell corpses labeled by phagosomal markers was determined by dividing the number of labeled cell corpses by the total number of button-like cell corpses. The percentage of vacuoles in slc-36.1(yq110) embryos labeled with phagosomal markers was determined by dividing the number of labeled vacuolar structures by the total number of vacuolar structures. 15 embryos were scored for each strain.

Examination of embryo hatching ratio
To examine embryonic development, 20 young adult worms (24 h after larval stage 4 [L4]) were placed on a fresh seeded nematode growth media (NGM) plate for 2 h. The worms were then removed, and the eggs laid on the plate were counted. 250 embryos were quantified for each strain. At least three independent experiments were performed. Amino acid supplementation was performed by including free amino acids (Ameresco) at 100 mM in both NGM plates and OP50 culture containing 7.5 µl ligand lysate and 2.5 µl target lysate expressing EGFP–SLC-36.1, EGFP–Myc–His–PPK-3, or Myc–His were lysed in 0.5 ml lysis buffer (10 mM Tris/Cl, pH 7.5, 150 mM NaCl; 0.5 mM EDTA; 0.5% NP-40, phosSTOP [Roche], and protease inhibitor cocktail [Roche]). Total protein concentrations of these cell lysates were measured with NanoDrop One (Thermo Fisher Scientific). Cell lysates were diluted with lysis buffer to a final concentration of 3.5 µg/µl. The fluorescent signals of the target proteins were examined, and no absorption, aggregation, or fluorescence variation was found. The signals of EGFP–SLC-36.1 and EGFP were well above the detection limit of the Monolith NT.115 instrument (NanoTemper Technologies). 10 µl of a mixture containing 7.5 µl ligand lysate and 2.5 µl target lysate expressing EGFP–SLC-36.1 or EGFP was loaded into NT.115 standard coated capillaries (NanoTemper Technologies). A series of cell lysates containing Myc–His–PPK-3 (0, 0.5, 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5 µl) was used as ligands to build a ligand/target gradient. Lysates of Myc–His vector–expressing cells were used as a supplement to ensure

Phagolysosome tubulation events and phagolysosome area were measured manually based on the projected image using softWoRx. 15 corpses were scored for each genotype. For more detailed monitoring, pre-comma embryos with 10 Z-section images (0.5 µm/section) were captured every 10 s for 20 min. To monitor ALR, adult worms (24 h after L4) were anesthetized in 3 µl M9 buffer containing 1 mM levamisole and covered with a 2% agar pad in a glass-bottom dish (MatTek). Posterior germline U-turn was marked. 10 Z-section images (0.35 µm/section) of the hypodermis in each genotype were captured every 5 s for 10 min. Images were deconvoluted and projected as above. The excitation filters used for GFP and mCh in all images were 488 and 559 nm, respectively.

Quantification of lysosomes in ABplaapppp cells
Embryos were separated in 3 µl egg salt buffer and covered with a 2% agar pad in a glass-bottom dish (MatTek). Comma-stage embryos were marked, and ABplaapppp cells containing LAAT-1::GFP–labeled phagolysosomes were captured with a Z-series of 0.2 µm/section for a total of 26 sections. LAAT-1::GFP–positive lysosome particles in ABplaapppp cells were counted. 40 or more embryos were analyzed for each genotype.

Quantification of hypodermal lysosome sizes
Adult worms (30 h after L4 or 24 h after L4 + 6 h starvation) were anesthetized in 3 µl M9 buffer containing 1 mM levamisole. Images of posterior hypodermis were captured. ZEN 2 software was used to automatically mark NUC-1::mCh–positive lysosomes and measure the area. Five worms were analyzed and >100 lysosomes were measured for each genotype.

MST protein–protein binding assay
MST was used to characterize protein interactions as described previously (Seidel et al., 2013; Asmari et al., 2018). Briefly, HEK293T cells were cultured at 37°C with 5% CO2 in DMEM supplemented with 10% FBS (HyClone), 100 U/ml penicillin, and 100 mg/ml streptomycin. Transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. HEK293T cells overexpressing EGFP–SLC-36.1, EGFP, Myc–His–PPK-3, or Myc–His were lysed in 0.5 ml lysis buffer (10 mM Tris/Cl, pH 7.5, 150 mM NaCl; 0.5 mM EDTA; 0.5% NP-40, phosSTOP [Roche], and protease inhibitor cocktail [Roche]). Total protein concentrations of these cell lysates were measured with NanoDrop One (Thermo Fisher Scientific). Cell lysates were diluted with lysis buffer to a final concentration of 3.5 µg/µl. The fluorescent signals of the target proteins were examined, and no absorption, aggregation, or fluorescence variation was found. The signals of EGFP–SLC-36.1 and EGFP were well above the detection limit of the Monolith NT.115 instrument (NanoTemper Technologies). 10 µl of a mixture containing 7.5 µl ligand lysate and 2.5 µl target lysate expressing EGFP–SLC-36.1 or EGFP was loaded into NT.115 standard coated capillaries (NanoTemper Technologies). A series of cell lysates containing Myc–His–PPK-3 (0, 0.5, 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5 µl) was used as ligands to build a ligand/target gradient. Lysates of Myc–His–PPK-3 expressing cells were used as a supplement to ensure
each reaction contained a similar amount of Myc-His proteins. MST measurements were performed at 25°C, 20% excitation power, and medium MST power. All experiments were repeated four times for each measurement. Data analyses were performed using NanoTemper analysis software.

Recombinant proteins and GST pull-down
Recombinant GST–SLC-36.1(1–53) and GST–SLC-36.1(101–142) proteins were expressed in Rosetta (DE3) bacterial cells and purified with glutathione-Sepharose beads (GE Healthcare) according to the instructions provided by the supplier. 35S-labeled Myc–His–PPK-3(1–668) and Myc–His–PPK-3(669–1497) were prepared by in vitro translation using TNT T7-Coupled Reticulocyte Lysate System (Promega). Purified GST, GST–SLC-36.1(1–53), and GST–SLC-36.1(101–142) proteins (5 mg of each) immobilized on glutathione-Sepharose beads were preincubated with 2% BSA in binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% NP-40, and 1 mM PMSF) for 1 h, and then incubated with 35S-labeled Myc–His–PPK-3(1–668) and Myc–His–PPK-3(669–1497) in binding buffer containing 1% BSA at 4°C overnight. The resins were washed gently three times in washing buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1% NP-40, and PMSF). Bound proteins were resolved on SDS-PAGE and visualized by autoradiography.

RNAi
The bacteria feeding protocol was used in RNAi experiments. L4 larvae (PO) were cultured on the RNAi plates. The F1 progeny at the L4 stage were transferred to fresh RNAi plates and aged for 24 h. Adult F1 progeny were cultured for another 6 h on seeded or unseeded RNAi plates before the hypodermis was examined.

HVEM analysis
Adult worms (30 h after L4) were rapidly frozen using a high-pressure freezer (EM PACT2; Leica Biosystems). For embryos, 20 young adults (24 h after L4) were transferred to unseeded NGM plates. Worms were removed after 6 h, and embryos were harvested and rapidly frozen. Freeze substitution was performed in anhydrous acetone containing 1% osmium tetroxide. The samples were kept sequentially at −90°C for 72 h, −60°C for 8 h, and −30°C for 8 h and were finally brought to 20°C for 10 h in a freeze-substitution unit (EM AFS2; Leica Biosystems). The samples were washed three times (1 h each time) in fresh anhydrous acetone and were gradually infiltrated with Embed-812 resin in the following steps: resin/acetone 1:3 for 3 h, 1:1 for 5 h, 3:1 overnight, and 100% resin for 4 h. Samples were then kept overnight and embedded at 60°C for 48 h. The fixed samples were cut into 70-nm sections with a microtome EM UC7 (Leica Biosystems) and electron-stained with uranyl acetate and lead citrate. Sections were observed with a JEM-1400 (JEOL) operating at 80 kV.

Statistical analysis
Data were analyzed with Prism (GraphPad Software) or Excel (Microsoft Office) to generate curves or bar graphics. Error bars represent SEM. The two-tailed unpaired t test was used for statistical comparison of two groups of samples. One-way ANOVA followed by Dunnett’s posttest was performed for statistical comparison of multiple groups of samples. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Online supplemental material
Fig. S1 analyzes SLC-36.1 expression pattern and subcellular localization in coelomocyte. Fig. S2 analyzes the dynamic change of His-24::GFP- and LAAT-1::mCh–positive phagolysosomes in N2 and slc-36.1(yq110) embryos, and lysosomal degradation of GFP::CAV-1, RME-2::GFP, and VIT-2::GFP in N2, slc-36.1(lf) and laat-1(qx42) embryos. Fig. S3 characterizes lysosome reformation from phagolysosomes in the N2 embryo. Fig. S4 shows that enlarged lysosomes in slc-36.1(yq110) hypodermis are positive for GFP::LGG-1(qx42), and ppk-3(n2668) embryos. Videos 6, 7, and 8 show time-lapse monitoring of phagolysosomal dynamics in N2, slc-36.1(yq110), and ppk-3(n2668) adult hypodermis. Table S1 summarizes the WormBase IDs of mutants and transgenic arrays. Table S2 lists C. elegans, bacterial, and mammalian expression vectors. Table S3 lists primers used for GFP knock-in by CRISPR/Cas9.

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