Caenorhabditis elegans HOPS and CCZ-1 mediate trafficking to lysosome-related organelles independently of RAB-7 and SAND-1

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ABSTRACT As early endosomes mature, the SAND-1/CCZ-1 complex acts as a guanine nucleotide exchange factor (GEF) for RAB-7 to promote the activity of its effector, HOPS, which facilitates late endosome–lysosome fusion and the consumption of AP-3–containing vesicles. We show that CCZ-1 and the HOPS complex are essential for the biogenesis of gut granules, cell type–specific, lysosome-related organelles (LROs) that coexist with conventional lysosomes in Caenorhabditis elegans intestinal cells. The HOPS subunit VPS-18 promotes the trafficking of gut granule proteins away from lysosomes and functions downstream of or in parallel to the AP-3 adaptor. CCZ-1 also acts independently of AP-3, and ccz-1 mutants mis-traffick gut granule proteins. Our results indicate that SAND-1 does not participate in the formation of gut granules. In the absence of RAB-7 activity, gut granules are generated; however, their size and protein composition are subtly altered. These observations suggest that CCZ-1 acts in partnership with a protein other than SAND-1 as a GEF for an alternate Rab to promote gut granule biogenesis. Point mutations in GLO-1, a Rab32/38-related protein, predicted to increase spontaneous guanine nucleotide exchange, specifically suppress the loss of gut granules by ccz-1 and glo-3 mutants. GLO-3 is known to be required for gut granule formation and has homology to SAND-1/Mon1–related proteins, suggesting that CCZ-1 functions with GLO-3 upstream of the GLO-1 Rab, possibly as a GLO-1 GEF. These results support LRO formation occurring via processes similar to conventional lysosome biogenesis, albeit with key molecular differences.

INTRODUCTION Many cell types contain multiple types of lysosomal compartments. Like the ubiquitous conventional lysosomes with which they typically coexist, lysosome-related organelles (LROs) are often acidified and produced through the action of the endocytic pathway (Marks et al., 2013; Yousefian et al., 2013). LROs represent a diverse array of compartments that are distinguished from each other by their cell type–specific morphology, composition, and function (Dell’Angelica et al., 2000). Defective formation of LROs can lead to the disease Hermansky–Pudlak syndrome (HPS). HPS patients and mouse HPS disease models have altered body pigmentation and blood clotting, resulting from aberrant biogenesis of LROs in melanocytes and platelets (Huizing et al., 2008). LROs are not restricted to vertebrates and are found in invertebrates, protozoa, and possibly plants (Raposo et al., 2007; Sohn et al., 2007; Docampo et al., 2010).

Gut granules are cell type–specific LROs found within Caenorhabditis elegans intestinal cells (Hermann et al., 2005). They contain birefringent and autofluorescent material, whose accumulation is coincident with their formation during embryogenesis (Lauber et al., 1980; Bossinger and Schierenberg, 1992). Gut granules function in zinc storage, limiting its toxicity (Roh et al., 2012), contain tryptophan...
derivatives of the kynurenine pathway (Coburn et al., 2013), and are linked to lipid metabolism (Mak et al., 2006; O'Rourke et al., 2009; O'Rourke and Ruvkun, 2013). Although gut granules share some characteristics and proteins with conventional lysosomes, the two organelles are distinct and coexist within intestinal cells (Clokey and Jacobson, 1986; Levitte et al., 2010; Hermann et al., 2012). Many of the factors mediating trafficking to C. elegans gut granules have evolutionarily conserved roles in the biogenesis of LROs in Drosophila and mammals (Hermann et al., 2005, 2012; Raposo et al., 2007).

Most of the molecular machinery known to guide LRO biogenesis is also used in the formation of conventional lysosomes. These include the AP-3 adaptor (Dell'Angelica, 2009), BLOC-1 (Di Pietro et al., 2006; Salazar et al., 2006; Setty et al., 2007), and HOPS tethering complexes (Zatic et al., 2011a; Solinger and Spang, 2013), suggesting that the mechanisms promoting trafficking to LROs and lysosomes are likely to be similar. However, there are some factors, such as Rab32/38-related proteins (Ma et al., 2004; Hermann et al., 2005; Wasmiejer et al., 2006), that likely function exclusively in the biogenesis of LROs. Knowing the similarities and differences between trafficking pathways leading to lysosomes and LROs will be key in understanding the biogenesis of LROs and the cellular processes that simultaneously generate these related, yet structurally and functionally distinct organelles.

Studies reveal an evolutionarily conserved mechanism for protein trafficking from early endosomes to lysosomes by which early endosomes, or early endosome subdomains (Rink et al., 2005; Vonderheft and Helenius, 2005), mature into late endosomes via the action of a SAND-1(Mon1)/CCZ-1 complex. This complex promotes both the inactivation and removal of RAB5 from early endosomes and the activation and recruitment of RAB7, a process called Rab conversion (Kinchen and Ravichandran, 2010; Nordmann et al., 2010; Poteryaev et al., 2010; Gerondopoulos et al., 2012). Active RAB7 recruits the HOPS tethering complex to late endosomes and lysosomes (Cabrera et al., 2010; Ostrowicz et al., 2010), promoting their heterotypic fusion to create endolysosomes (Caplan et al., 2001; Siriam et al., 2003; Richardson et al., 2004; Kinchen et al., 2008; Hickey and Wickner, 2010; Pols et al., 2013), which then mature into lysosomes via membrane budding (Pryor et al., 2000; Bright et al., 2005). HOPS also functions in the fusion of Golgi apparatus or early endosome–derived, AP-3–containing vesicles with endolysosomes and lysosomes (Darsow et al., 2001; Angers and Merz, 2009; Cabrera et al., 2010).

Parallel with lysosome biogenesis suggest that similar mechanisms function in trafficking to LROs. Mammalian paralogues of the SAND-1 and CCZ-1, called HPS1 and HPS4 (Hoffman-Sommer et al., 2005; Kinch and Grishin, 2006), respectively, form BLOC-3 (Chiang et al., 2003; Martina et al., 2003; Nazarian et al., 2003), which functions in the formation of LROs (Oh et al., 1996; Suzuki et al., 2002). Rab7 is localized on immature and mature LROs (Jordens et al., 2006; Zhang et al., 2011; Ambrosio et al., 2012), and disrupting Rab7 activity can alter the distribution of LRO contents (Hirosaki et al., 2002; Kawakami et al., 2008; Hida et al., 2011). In various metazoan systems, mutations affecting the function of HOPS subunits cause reduced numbers of LROs (Sevnioukov et al., 1999; Hermann et al., 2005; Pulipparacharuvil et al., 2005; Sadler et al., 2005; Maldonado et al., 2006; Akbar et al., 2009; Thomas et al., 2011). AP-3 functions during the formation of LROs (Ooi et al., 1997; Simpson et al., 1997; Kantheti et al., 1998; Dell’Angelica et al., 1999; Feng et al., 1999; Mullins et al., 1999, 2000; Kretzschmar et al., 2000; Hermann et al., 2005) by interacting with the cytoplasmic domain of a subset of LRO–destined transmembrane proteins, directing their vesicular transport to LROs from early endosomes (Huizing et al., 2001; Theos et al., 2005; Setty et al., 2007).

Here we systematically analyze the role of genes promoting protein trafficking from early endosomes to lysosomes during gut granule biogenesis in C. elegans intestinal cells and document genetic interactions revealing their functional relationships. Our results suggest that the pathways constructing LROs and conventional lysosomes are likely similar; however, they highlight significant molecular differences.

**RESULTS**

**Formation of autofluorescent and birefringent gut granules in endolysosomal trafficking mutants**

To address whether the machinery that mediates protein trafficking from early endosomes to conventional lysosomes also affects trafficking to LROs, we used C. elegans mutants that lack or have strongly reduced activity of VPS-C/HOPS, CCZ-1, SAND-1/Mon1, and RAB-7. These mutants were analyzed for their effects on the formation of gut granules, which contain birefringent material and autofluorescent material and are acidified (Lauffer et al., 1980; Clokey and Jacobson, 1986; Bossinger and Schierenberg, 1992). Defective gut granule biogenesis leads to loss of acidified intestinal organelles and loss and/or extracellular misaccumulation of the birefringent and autofluorescent material, which we refer to as a gut granule loss (Glo) phenotype (Hermann et al., 2005). Following standard C. elegans nomenclature, genes are given in lowercase italics and proteins are given in uppercase nonitalic.

**VPS-C/HOPS.** VPS-C comprises VPS-11, -16, -18, and -33. Two additional subunits, VPS-39 and -41, associate with VPS-C to form HOPS (Nickerson et al., 2009; Balderhaar and Ungermann, 2013; Graham et al., 2013). C. elegans encodes a protein orthologous to each HOPS subunit (Solinger and Spang, 2013; Supplemental Figure S1), and there are mutants predicted to severely disrupt the function of each of the six HOPS subunits (Supplemental Figure S1). Our prior work showed that mutations disrupting vps-16 or vps-41 led to a Glo phenotype in adults (Hermann et al., 2005), and we found that additional mutant alleles and RNA interference (RNAi) targeting these genes led to a similar phenotype, further supporting their role in gut granule biogenesis (Figure 1 and Table 1). We investigated whether the four other subunits of VPS-C/HOPS were similarly required for gut granule biogenesis. Mutations in three other HOPS genes—vps-11, vps33.1, and vps-39—also led to a reduction in the number of autofluorescent compartments (Figure 1 and Table 1). Of these, vps-39 presented the weakest Glo phenotype, which was enhanced by vps-39(RNAi) (Table 1), suggesting that the vps-39 mutant allele retains function or the adult Glo phenotype can be maternally rescued. The vps-18(tm1125) mutant did not display an obvious alteration in adult gut granule number (Figure 1 and Table 1), although it did exhibit defective formation of gut granules in embryos (see later discussion).

The VPS-C/HOPS subunit mutants that exhibited an adult Glo phenotype also displayed maternal effect embryonic lethality, producing progeny that arrest at a stage in embryogenesis before the formation of gut granules, thus preventing an analysis of their effect on gut granule biogenesis in embryos (unpublished observations). We therefore used RNAi, which often leads to a partial knockdown of gene function, to assess the role of VPS-C/HOPS genes in embryonic gut granule biogenesis. Birefringent material, which can be visualized with polarization microscopy, is restricted to, and first becomes apparent within, gut granules at the bean stage, midway through embryogenesis (Lauffer et al., 1980). This marker continues
embryos displayed a strong and fully penetrant Glo phenotype (Table 2 and Figure 2P). At 22°C, 15°C, pretzel-stage the formation of embryonic gut granules (Figure 2E and Table 2). At 25°C, many vps-18 mutant embryos arrested before elongation, and 76% (n = 33) of these contained birefringent material distributed throughout the embryo (Figure 2Q), suggestive of altered formation or trafficking of birefringent material, which is normally restricted to intestinal cells (Hermann et al., 2005).

We carried out temperature-shift experiments to determine when vps-18 activity promotes the formation of birefringent gut granules. vps-18 mutant embryos cultured at the permissive temperature were shifted to the nonpermissive temperature during early to mid embryogenesis and then scored for the presence and localization of birefringent material at the pretzel stage. Consistent with VPS-18 functioning directly in the formation of gut granules, we found that nearly all of the vps-18 mutant embryos upshifted before the early bean stage, when birefringent gut granules first become apparent (Lauffer et al., 1980), lacked or displayed a significant reduction in the number of birefringent intestinal granules (Figure 2Q).

The vps-18(tm1125) allele introduces a premature stop codon predicted to truncate the C-terminal 760 amino acids of VPS-18, which contains the α-solenoid, coiled-coil, and RING finger domains (Xiao et al., 2009; Supplemental Figure 1), suggesting that this allele may completely disrupt vps-18(+) activity. However, we found that application of vps-18 RNAi to vps-18(tm1125) mutants led to a significant decrease in the number of birefringent granules (Table 1). Thus the vps-18(tm1125) mutant allele exhibits significant activity in promoting adult gut granule formation. In contrast, vps-18(tm1125) mutant embryos exhibited a fully penetrant Glo phenotype at 22°C (Table 2), suggesting that vps-18 function is severely reduced at this stage. We therefore carried out our analyses of vps-18 activity in embryos.

We first addressed whether the defect in gut granule formation exhibited by the vps-18 mutant extended beyond just the generation and localization of birefringent material. Gut granules are the major acidified compartments within embryonic intestinal cells and are stained by LysoSensor Green, whose accumulation and fluorescence depend on an acidic pH (Levitte et al., 2010). Whereas Lyso-Sensor Green marked wild-type gut granules, organelles within vps-18 mutant intestinal cells were not stained, indicating a general defect in gut granule biogenesis (Figure 2, F and J).

In metazoans, there are alternate VPS-16 and VPS-33 subunits that associate with VPS-C/HOPS (Zilatic et al., 2011a; Solinger and Spang, 2013), which are necessary for the formation of some LROs (Lo et al., 2005; Urban et al., 2012). In C. elegans, SPE-39 is homologous to VPS-16, and VPS-33.2 and VPS-45 are homologous to VPS-33.1 (Solinger and Spang, 2013). Mutations in spe-39 or vps-45 and RNAi targeting spe-39, vps-33.2, or vps-45 did not

FIGURE 1: Autofluorescent compartments in adult intestinal cells. The 9-μm maximum-intensity projections, which span 50% the width of the intestine, of GFP channel autofluorescence centered on the lumen of the anterior intestine show that, in comparison to (A) wild type, the (B) vps-11, (C) vps-16, (E) vps-33, (F) vps-39, (G) vps-41, and (I) ccz-1 mutants exhibited reduced numbers of autofluorescent compartments. Mutations in (D) vps-18, (H) sand-1, and (J) rab-7 did not obviously alter the number of autofluorescent organelles. Black arrows denote representative autofluorescent compartments.

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**TABLE 1:** Gut granules in adults.

Continues
lead to an adult Glo phenotype (Table 1). The Arf-like GTPase Arl8 physically interacts with VPS41 in mammals, and mutation of the C. elegans orthologue arl-8 phenocopies vps-18 mutants (Xiao et al., 2009; Nakae et al., 2010; Garg et al., 2011), suggesting that it functions with the HOPS complex. However, we did not see any effect of an arl-8 mutation on the formation of autofluorescent gut granules (Table 1). In yeast, the VPS-C subunits can also associate with VPS-3 and VPS-8, instead of VPS-39 and VPS-41, to generate CORVET (Pepowska et al., 2007). There is no obvious VPS-3 homologue in C. elegans (Solinger and Spang, 2013). Mutations or RNAi targeting vps-8(jc24C1.4) adult progeny of vps-41(j+) apt-6(j+) apt-7(j+) parents were identified by the accumulation of apoptotic corpses in the germline and arrested embryos in the uterus. rab-7(j+)/rab-7(j+) adult progeny of rab-7(j+)/+ glo(j)/glo(j) parents were identified by the refractive structures visible with DIC microscopy in the most proximal oocytes, which are indicative of rab-7(j+) adults. According to published data on the effects of Glo proteins, we can conclude that the HOPS complex forms in the formation of gut granules.

### TABLE 1: Gut granules in adults. Continued

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All strains were grown at 22°C unless otherwise noted. Adult-stage animals were analyzed using fluorescence microscopy with a GFP or DAPI filter set and were scored for the number of autofluorescent organelles within the anterior intestine located between the pharynx and vulva.

**Non-GFP-expressing animals, which lack jcpEx2, were Glo (unpublished data).**

*Wild type had >200 at both 22 and 25°C.*

**RNAi was carried out in the nT1[qIs51] strain (Simmer et al., 2003).** F33E2.4(RNAi) targeting a gene not involved in gut granule formation did not alter number of autofluorescent compartments in nT1(qIs51).

**vps-45(tm246) animals were grown at 22°C from early larvae to adulthood before scoring.**

**vps-39(tm2253)/nT1[qIs51] animals were placed on RNAi plates as embryos and grown for two generations before being scored. Only animals lacking GFP, which are vps-39(j+)/vps-39(j+), were analyzed.** F33E2.4(RNAi) targeting a gene not involved in gut granule formation did not alter number of autofluorescent compartments in these animals (unpublished data).**

**Dpy progeny of dpy-11(e224) ccz-1(t2129); jcpEx2[ccz-1::yfp; myo-2::gfp] animals expressing GFP were scored.** Non-GFP-expressing animals, which lack jcpEx2, were Glo (unpublished data).

**RNAi was carried out in the N2 background.** F33E2.4(RNAi) targeting a gene not involved in gut granule formation did not alter number of autofluorescent compartments.

**Non-GFP-expressing arl-8(j+)/arl-8(j+) progeny of arl-8(m2504);nT1[qIs51] were scored.**

**Non-GFP-expressing rab-7(j+)/rab-7(j-) progeny of rab-7(ok511);mln1[mln14] were scored.**

**Adults scored had prominent, enlarged, refractile structures visible with DIC microscopy in the most proximal oocytes, which are indicative of rab-7(j-).**

**Unc-4(e120), which did not alter the Glo phenotype, was present in the background.**

**vps-41(j+)/vps-41(j-) adult progeny of vps-41(j+); apt-6(j+)/apt-6(j-) parents were identified by the accumulation of apoptotic corpses in the germline and arrested embryos in the uterus.**

**rab-7(j+)/rab-7(j-) adult progeny of rab-7(j+)/+ glo(j)/glo(j) parents were identified by the refractive structures visible with DIC microscopy in the most proximal oocytes, which are indicative of rab-7(j-).**

**sand-1/Mon1 and ccz-1.** Current models for membrane trafficking to conventional lysosomes in C. elegans point to a role for a complex of SAND-1 and CCZ-1 as regulators of early endosome–to–late endosome maturation and Rab-5–to–Rab-7 conversion (Kinchen and Ravichandran, 2010; Nieto et al., 2010; Poteryaev et al., 2010). Mammalian genomes encode three distinct proteins—Mon1a, Mon1b, and HPS1—that are homologous to SAND-1 and two distinct proteins—Ccz1 and HPS4—that are homologous to CCZ-1. HPS1 and HPS4 function in the formation of LROs (Oh et al., 1996; Suzuki et al., 2002). The genome of C. elegans encodes only single proteins that are highly homologous to SAND-1/Mon1 and CCZ-1.

We found that ccz-1(f2129) and ccz-1(ok2182), two different mutant alleles of ccz-1 that lack or have severely reduced activity (Nieto et al., 2010), respectively, similarly resulted in the loss of birefringent gut granules and mislocalization of birefringent material into the intestinal lumen (Figure 2B and Table 2). Consistent with altered gut...
FIGURE 2: Analysis of birefringent and acidified gut granules in embryonic intestinal cells. Embryos were analyzed with polarization microscopy to detect birefringent material, stained with LysoSensor Green, and analyzed with fluorescence microscopy to detect acidified compartments. (A, F) Wild-type, (C, H) sand-1, and (D, I) rab-7 mutant embryos displayed birefringent and acidified gut granules (white arrows). (B, G) ccz-1 and (E, J) vps-18 mutants lacked acidified and birefringent gut granules and mislocalized birefringent material into the intestinal lumen (black arrows). (K–O) RNAi targeting subunits of the VPS-C/HOPS complex in an rrf-3(pk1426) RNAi–sensitive strain caused a reduction in the number of birefringent organelles (white arrows) and sometimes led to misaccumulation of birefringent material in the intestinal lumen (black arrows). (P, Q) Birefringent material (white arrows) was present within the intestinal cells of vps-18 mutants at 15°C and was distributed throughout the embryo at 25°C. The intestine is flanked by black arrowheads in A–P. Pretzel-stage embryos are shown in A–C, E, and K–P; 1.5-fold-stage embryos are shown in D and F–J. A terminally arrested pre–bean stage vps-18 mutant embryo is shown in Q. Embryos are ∼50 μm in length. (R) Individual vps-18 mutant embryos were shifted from 15 to 22°C at the indicated stage, and on reaching threefold stage or later, were
granule formation, ccz-1 mutant embryos lacked LysoSensor Green–stained compartments (Figure 2G). ccz-1 mutants also lacked or had substantially reduced numbers of autofluorescent gut granules in adults (Figure 1I and Table 1). The Glo phenotypes exhibited by the ccz-1(12129) mutant were rescued by the introduction of ccz-1(+):yfp (Tables 1 and 2). In contrast to ccz-1 mutants, the strong loss-of-function sand-1(or552) mutant and the likely null sand-1(ok1963) mutant (Poteryaev et al., 2007) did not exhibit a loss of birefringent, acidified, or autofluorescent gut granules (Figures 1H and 2, C and H, and Tables 1 and 2), suggesting that SAND-1 does not play a significant role in gut granule biogenesis.

**rab-7.** *C. elegans* codes for a single RAB-7–related protein, which is orthologous to Rab7 in other organisms (Gallegos et al., 2012). In *C. elegans*, the SAND-1/CCZ-1 complex promotes association of RAB-7 with endosomal membranes (Poteryaev et al., 2007; Kinchen and Ravichandran, 2010; Nieto et al., 2010), likely by acting as a RAB-7 guanine nucleotide exchange factor (GEF; Nordmann et al., 2010; Gerondopoulos et al., 2012). The HOPS complex is an effector of RAB-7 in yeast and possibly other organisms (Nickerson et al., 2009; Epp et al., 2011; Zlatic et al., 2011a). Thus RAB-7 might act as a functional link between CCZ-1 and HOPS during trafficking to gut granules. If this is the case, rab-7 mutants should exhibit defects in gut granule biogenesis similar to ccz-1 and VPS-C/HOPS mutants.

The rab-7(ok511) null mutant exhibits maternal effect embryonic lethality and arrests at a stage after gut granules are normally formed (Yu et al., 2008), which allowed us to investigate the role of RAB-7 in the biogenesis of gut granules. Whereas the number of autofluorescent gut granules was not substantially reduced in rab-7 mutants (Figure 1J and Table 1), at the L4 stage their diameter was reduced by 65% relative to wild type (Supplemental Figure S2). The number of birefringent gut granules in rab-7 mutants was modestly reduced (Table 2); however, unlike ccz-1 and vps-18 mutants, birefringent material was never detected in the intestinal lumen (Figure 2D and Table 2). The average diameter of gut granules in 1.5-fold–stage embryos increased from 0.7 μm in wild type to 1.2 μm in rab-7 mutants (Supplemental Figure S2). Gut granules in rab-7 mutant embryos were stained by LysoSensor Green (Figure 2I), suggesting that they are acidified. Together these results indicate that loss of rab-7 function only subtly affects gut granule formation.

**Protein trafficking to gut granules**

The loss or misaccumulation of gut granule contents might result from specific defects in the formation/trafficking of birefringent or autofluorescent material or more generally from alterations in gut granule biogenesis. In addition, alterations in protein trafficking to gut granules might not obviously affect the localization of these materials.

To address these possibilities, we analyzed the localization of gut granule proteins in the endolysosomal trafficking mutants. CDF-2 and PGP-2 are multipass transmembrane proteins, which at steady state exclusively localize to the gut granule (Figure 3, A and B; Davis et al., 2009; Hermann et al., 2012). The single-pass membrane-associated protein LMP-1 localizes to both gut granules and conventional lysosomes (Hermann et al., 2012). LMP-1 transport to gut granules depends on the activity of the AP-3 adaptor complex, whereas CDF-2:green fluorescent protein (GFP) and PGP-2 can be trafficked to gut granules independently of AP-3 (Hermann et al., 2012). We carried out trafficking studies in 1.5-fold embryos, a stage when both gut granules and these proteins are initially being synthesized within the 20 newly polarized intestinal epithelial cells (Hermann et al., 2005).

Similar to their effects on the localization of birefringent and autofluorescent material, the colocalization of CDF-2::GFP and PGP-2 at gut granules was not disrupted in sand-1 and rab-7 mutants (Figure 3, J–P). sand-1 mutants did not substantially reduce the localization of LMP-1 to gut granules containing CDF-2::GFP and PGP-2 (Figures 4, J–L, and 5, J–L). In contrast, in rab-7 mutant embryos, LMP-1 levels were significantly reduced and many CDF-2::GFP and PGP-2 compartments lacked detectable LMP-1 (Figures 4, M–P, and 5, M–P). These data indicate that gut granules are formed in sand-1 and rab-7 mutants. However, RAB-7 activity promotes the normal accumulation of LMP-1 at the gut granule.

Indicative of a significant defect in gut granule biogenesis, the distribution and colocalization of gut granule proteins were dramatically altered by mutations in vps-18 and ccz-1. PGP-2 and CDF-2::GFP colocalized at gut granules in wild-type 1.5-fold-stage embryos (Figure 3, A–C and P). In vps-18 mutants, CDF-2::GFP was enriched on organelles that did not contain PGP-2 (Figure 3, D–F and P). In ccz-1 mutants, CDF-2::GFP was localized to compartments that typically lacked PGP-2, which could be only weakly detected on intestinal organelles (Figure 3, G–I and P). LMP-1, which normally colocalized with PGP-2 on gut granules in wild type (Figure 4, A–C and P), rarely colocalized with PGP-2 in both vps-18 and ccz-1 mutants (Figure 4, D–I and P). CDF-2::GFP compartments in wild type contained LMP-1 (Figure 5, A–C and P), whereas a reduced proportion of CDF-2::GFP compartments contained LMP-1 in vps-18 and ccz-1 mutants (Figure 5, D–I and P).

Our results indicate that one or more gut granule–associated proteins are mislocalized in embryos deficient for VPS-18 or CCZ-1 function. PGP-2 exhibited weak staining in vps-18 and ccz-1 mutants, and LMP-1 normally associates with both gut granules and lysosomes. We therefore examined the localization of CDF-2::GFP in strains with altered VPS-18 and CCZ-1 activity. In vps-18 mutant embryos, CDF-2::GFP was often associated with organelles located near the apical surface of intestinal cells (compare Figure 6, A and D), which is where conventional lysosomes and early endosomes are typically localized (Hermann et al., 2012). In contrast, CDF-2::GFP–containing compartments were distributed throughout the cytoplasm of ccz-1 mutants (Figure 3G). In wild-type and both mutant strains, CDF-2::GFP did not accumulate on RAB-5–marked early endosomes (Supplemental Figure S3). Of note, CDF-2::GFP–containing organelles in vps-18 mutants were often marked by Rab-7, unlike ccz-1 mutants and wild type (Figure 6, A–I and S). Rab-7 was often associated with endolysosomes marked by LMP-1::GFP (Figure 7, F–H, and Supplemental Figure S2). Consistent with CDF-2::GFP being mislocalized to lysosomes in vps-18 embryos, a Glo phenotype only 9% of the time (n = 33). Wild-type embryos that underwent similar temperature shifts always displayed >50 birefringent granules.
### Genotype

<table>
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<th>Genotype</th>
<th>Percentage lacking birefringence in intestinal cells</th>
<th>Percentage with 1–20 birefringent granules in intestinal cells</th>
<th>Percentage with 21–50 birefringent granules in intestinal cells</th>
<th>Percentage with &gt;50 birefringent granules in intestinal cells</th>
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All strains were grown at 22°C unless otherwise noted. Threefold- and later-stage embryos were analyzed using polarization microscopy and scored for the presence and localization of birefringent material in the intestine.

a Percentage that also mislocalized birefringent material into the intestinal lumen are in parentheses.

b The phenotype of wild type was not altered by growth at 15°C (unpublished data).

c Dpy progeny of dpy-11(e224) ccz-1(t2129)/nT1[qIs51] were isolated, and their progeny were scored. dpy-11(e224) ccz-1(t2129); jcpEx2[ccz-1::yfp; myo-2::gfp] embryos expressing GFP were scored. Non-GFP-expressing animals, which lacked jcpEx2, were often Glo (not shown).

d Non-GFP-expressing embryos, which lacked jcpEx2, were often Glo (not shown).

f Non-GFP-expressing embryos, which lacked jcpEx2, were often Glo (not shown).

g Non-GFP-expressing embryos, which lacked jcpEx2, were often Glo (not shown).

h RNAi was carried out in an otherwise wild-type background. F33E2.4(RNAi) targeting a gene not involved in gut granule formation did not alter the number of birefringent compartments in the indicated strain. All embryos scored displayed numerous enlarged refractile structures visible with DIC microscopy, indicative of rab-7(-).

i Non-GFP-expressing embryos, which lacked jcpEx2, were often Glo (not shown).

j RNAi was carried out in the rrf-3(pk1426) RNAi–sensitive strain (Simmer et al., 2003). F33E2.4(RNAi) targeting a gene not involved in gut granule formation did not alter number of birefringent compartments in rrf-3(-).

k RNAi was carried out in the rrf-3(pk1426) RNAi–sensitive strain (Simmer et al., 2003). F33E2.4(RNAi) targeting a gene not involved in gut granule formation did not alter number of birefringent compartments in rrf-3(-).

l RNAi was carried out in the rrf-3(pk1426) RNAi–sensitive strain (Simmer et al., 2003). F33E2.4(RNAi) targeting a gene not involved in gut granule formation did not alter number of birefringent compartments in rrf-3(-).

m RNAi was carried out in the rrf-3(pk1426) RNAi–sensitive strain (Simmer et al., 2003). F33E2.4(RNAi) targeting a gene not involved in gut granule formation did not alter number of birefringent compartments in rrf-3(-).

n RNAi was carried out in the rrf-3(pk1426) RNAi–sensitive strain (Simmer et al., 2003). F33E2.4(RNAi) targeting a gene not involved in gut granule formation did not alter number of birefringent compartments in rrf-3(-).

TABLE 2: Gut granules in embryos.
FIGURE 3: The distribution of CDF-2::GFP and PGP-2 is altered in vps-18 and ccz-1 mutants. Anti–PGP-2 antibodies and ectopically expressed CDF-2::GFP colocalized at gut granules in (A–C) wild type and (J–L) sand-1 and (M–O) rab-7 mutants (black arrows within insets). (D–F) CDF-2::GFP–containing organelles in vps-18 mutants lacked PGP-2 staining (white arrows in insets). (G–I) The majority of CDF-2::GFP–labeled compartments in ccz-1 mutants lacked anti–PGP-2 staining (white arrows in insets). In A–O, 1.5-fold-stage embryos are shown, black arrowheads flank the intestine, and the insets are 5 μm wide. (P) For each genotype, at least 25 randomly selected CDF-2::GFP–containing intestinal compartments in five different 1.5-fold-stage embryos were scored for the presence of anti–PGP-2 signals. The mean is plotted, and error bars represent the 95% confidence limit. A one-way analysis of variance (ANOVA) comparing each mutant to wild type was used to calculate \( p \) values (\( ** p \leq 0.001 \)).
The distribution of PGP-2 and LMP-1 is altered in vps-18, ccz-1, and rab-7 mutants. Antibodies recognizing PGP-2 and LMP-1 colocalized at gut granules in (A–C) wild type and (J–L) sand-1 mutants (black arrows in insets). (D–I) PGP-2–stained organelles in vps-18 and ccz-1 mutants lacked LMP-1 staining (white arrows within insets). (M–O) In rab-7 mutants, LMP-1 staining was weakly present on some (black arrows in insets) but not all (white arrows in insets) anti-PGP-2–marked gut granules. In A–O, 1.5-fold-stage embryos are shown, black arrowheads flank the intestine, and insets are 5 μm wide. (P) For each genotype, at least 25 randomly selected PGP-2–stained intestinal compartments in five different 1.5-fold-stage embryos were scored for the presence of LMP-1 staining. The mean is plotted, and error bars represent the 95% confidence limit. A one-way ANOVA comparing each mutant to wild type was used to calculate p values (*p ≤ 0.05, **p ≤ 0.001).
FIGURE 5: The distribution of CDF-2::GFP and LMP-1 is altered in vps-18, ccz-1, and rab-7 mutants. Nearly all CDF-2::GFP–containing organelles were marked by anti–LMP-1 antibodies in (A–C) wild-type and (J–L) sand-1 mutant cells (black arrows in insets). In (D–F) vps-18, (G–I) ccz-1, and (M–O) rab-7 mutants, a subset of CDF-2::GFP–marked organelles contained anti–LMP-1 staining (black arrows in insets), although most lacked LMP-1 (white arrows in insets). In A–L, 1.5-fold-stage embryos are shown, and in M–O, a late-bean-stage embryo is shown. In A–O, black arrowheads flank the intestine and insets are 5 μm wide. (P) For each genotype, at least 25 randomly selected CDF-2::GFP–containing intestinal compartments in five different 1.5-fold-stage embryos were scored for the presence of LMP-1 staining. The mean is plotted, and error bars represent the 95% confidence limit. A one-way ANOVA comparing each mutant to wild type was used to calculate \( p \) values (*\( p \leq 0.05 \), **\( p \leq 0.001 \)).
CDF-2::GFP colocalized with an mCherry-tagged form of the lysosomal glucosylceramidase F11E6.1a (Levitte et al., 2010; Figure 6, M-O and T). CDF-2::GFP did not appreciably associate with F11E6.1a::mCherry in wild-type or ccz-1(-) mutant embryos (Figure 6, P-R and T). The identity of CDF-2::GFP compartments in ccz-1 mutants is unclear, as they lack markers for gut granules, early endosomes, late endosomes, and lysosomes, whereas disrupting VPS-18 activity results in gut granule proteins being missorted toward conventional lysosomes.

**Formation of conventional endolysosomes**

In addition to analyzing the effects of the endolysosomal trafficking pathway mutants on gut granule biogenesis, we also examined their role in controlling the number and identity of conventional endolysosomes in the embryonic intestinal primordium. LMP-1::GFP marks conventional lysosomes that are localized near the intestinal cell apical domain of 1.5-fold embryos (Hermann et al., 2012). Wild-type embryos contained an average 12 LMP-1::GFP compartments in the four intestinal cells that make up intestinal rings 2 and 3, whereas vps-18 mutants displayed fourfold more, which were enriched near the apical cell surface (Figure 7, A, B, and R). Relative to wild type, ccz-1 mutants had a twofold increase in the number of LMP-1::GFP compartments, which were not restricted to the apical domain (Figure 7, C and R). The number of LMP-1::GFP organelles was only slightly altered in sand-1 and rab-7 mutants (Figure 7, D, E, and R).

Owing to effects of vps-18 and ccz-1 mutations on the number and localization of LMP-1::GFP-containing compartments, we analyzed the identity of LMP-1::GFP-containing compartments using antibodies to RAB-5 and RAB-7. LMP-1::GFP did not accumulate on RAB-5 marked early endosomes in wild type or in vps-18 and ccz-1 mutants (Supplemental Figure S3, J–R and T). Of note, unlike wild type, vps-18 mutant embryos displayed an increase in the proportion of LMP-1::GFP compartments that also contained RAB-7 (Figure 7, F–K and S).

---

**FIGURE 6:** CDF-2::GFP is mislocalized to endolysosomes in vps-18 mutants. (A–C, G–I) Endogenous RAB-7 detected with anti–RAB-7 antibodies was not associated with CDF-2::GFP-containing organelles in wild type or ccz-1 mutants (white arrows in insets). (D–F) In vps-18 mutants, anti–RAB-7 staining was often localized to CDF-2::GFP–marked compartments (black arrows in insets). The lysosomal hydrolase F11E6.1a::mCherry did not appreciably localize to CDF-2::GFP–marked compartments in (J–L) wild type or (P–R) ccz-1 mutants (white arrows in insets). (M–O) In vps-18 mutants, F11E6.1a::mCherry often colocalized with CDF-2::GFP–containing compartments (black arrows in insets). In A–R, single optical sections of 1.5-fold-stage embryos are shown, black arrowheads flank the intestine, and insets are 5 μm wide. In A–F, white arrowheads denote the apical surface of intestinal cells. (S, T) For each genotype, at least 25 randomly selected CDF-2::GFP–containing intestinal compartments in five different 1.5-fold-stage embryos were scored for the presence of RAB-5 or F11E6.1a::mCherry. The mean is plotted, and error bars represent the 95% confidence limit. A one-way ANOVA comparing each mutant to wild type was used to calculate p values (*p ≤ 0.05, **p ≤ 0.001).
synchronously that defects in gut granule biogenesis in ccz-1 mutants, which are not present in sand-1 mutants, exacerbate effects on LMP-1::GFP localization, leading to the dramatic difference in LMP-1::GFP compartment number and subcellular localization in these two mutants.

**Interactions between AP-3 and endolysosomal trafficking factors in gut granule biogenesis**

The HOPS complex in yeast mediates the docking of AP-3–containing vesicles at the lysosome-like vacuole through direct interactions of AP-3 and a short region of VPS41, which is conserved in *C. elegans* VPS-41 (Angers and Merz, 2009; Cabrera et al., 2010).

**FIGURE 7:** Analysis of LMP-1::GFP trafficking. (A–E) Mutations in (B) vps-18, (C) ccz-1, and to a lesser extent (E) rab-7 led to an increased number of LMP-1::GFP–marked organelles. (R) The total number of LMP-1::GFP compartments was quantified in the four cells that compose Int2 and Int3 (marked by black arrows in A–E) in five different 1.5-fold-stage embryos of each genotype. The mean is plotted, and error bars represent the 95% confidence limit. A one-way ANOVA comparing each mutant to wild type was used to calculate *p* values (*p* ≤ 0.05, **p** ≤ 0.001). (F–H) Relative to wild type, a higher proportion of LMP-1::GFP compartments in (I–K) vps-18 mutants contained RAB-7 antibody signals, and a decreased proportion of LMP-1::GFP compartments in (L–N) ccz-1 and sand-1 mutants contained RAB-7 (in the insets, black arrows denote LMP-1::GFP compartments marked by RAB-7 antibodies and white arrows label compartments lacking a RAB-7 signal). (S) For each genotype, at least 25 randomly selected LMP-1::GFP–containing intestinal compartments in five different 1.5-fold-stage embryos were scored for the presence of signal from the RAB-7 antibody. The mean is plotted, and error bars represent the 95% confidence limit. A one-way ANOVA comparing each mutant to wild type was used to calculate *p* (*p* ≤ 0.05, **p** ≤ 0.001). In A–E, 1-μm maximum-intensity projections, and in F–Q, single optical sections, of 1.5-fold-stage embryos are shown. The white arrowhead in B denotes the apical cell membrane. In images showing embryos, the black arrowheads flank the intestine. The insets are 5 μm wide.
In mammalian cells, HOPS subunits colocalize and physically interact with AP-3 subunits (Zlatic et al., 2011b). The AP-3 adaptor complex has evolutionarily conserved roles in trafficking to LROs (Dell'Angelica, 2009); however, the functional relationship between AP-3 and HOPS in the biogenesis of LROs has not been investigated.

If VPS-C/HOPS activity is restricted to the same step as AP-3 during trafficking to gut granules, then AP-3 and VPS-C/HOPS mutants should have similar defects in gut granule biogenesis, and the mutants should not exacerbate each other's phenotypes. Inconsistent with this model, vps-18 mutant embryos completely lacked birefringent intestinal granules, whereas they were always present in null mutants of apt-6 and apt-7, which encode AP-3 β- and μ-subunits, respectively (Table 2). Moreover, the majority of AP-3; vps-18 double-mutant embryos exhibited a complete loss of birefringent gut granules, resembling the vps-18 mutant (Table 2). Single VPS-C/HOPS and AP-3 mutants similarly displayed reduced numbers of autofluorescent gut granules (Table 1). However, the vps-18 mutation, which did not exhibit decreased numbers of autofluorescent compartments on its own at 22°C, led to a complete loss of autofluorescent gut granules when introduced into both apt-6 and apt-7 mutant backgrounds (Table 1). A vps-41 mutation also enhanced the adult Glo phenotype of the apt-6 mutant (Table 1). Together these genetic interactions suggest that VPS-C/HOPS and AP-3 function at distinct steps in the biogenesis of gut granules.

We also examined whether CCZ-1 has functions distinct from AP-3 in trafficking to gut granules. The application of ccz-1(RNAi), which led to a moderate decrease in the number of autofluorescent gut granules on its own, significantly enhanced the Glo phenotype of apt-6 and apt-7 mutants (Table 1). Similarly, autofluorescent and birefringent compartments, which were always present in AP-3 mutants, were absent in an apt-7; ccz-1 double mutant (Tables 1 and 2). These results point to CCZ-1 having activities that are distinct from those of AP-3.

We extended our analyses of the interactions between mutations disrupting the function of AP-3, VPS-C/HOPS, and CCZ-1 by comparing their effects, alone or in combination, on the relative localization of two gut granule proteins, CDF-2::GFP and PGP-2. Whereas vps-18 mutants rarely displayed CDF-2::GFP compartments containing PGP-2 (Figures 3, D–F, and 8J), ~25% of CDF-2::GFP compartments were marked by PGP-2 in apt-7 and ccz-1 single mutants (Figures 3, G–I, and 8, A–C and J). We found that CDF-2::GFP compartments were more likely to contain PGP-2 in apt-7; vps-18 and apt-7; ccz-1 double mutants than the single mutants (Figure 8, D–J). The ability of an AP-3 mutant to modify the effects of mutations in vps-18 or ccz-1 on the colocalization of CDF-2::GFP and PGP-2 suggests that AP-3 functions upstream of, or parallel to, VPS-C/HOPS and CCZ-1 in trafficking to the gut granule.

We used AP-3 and other glo mutants that exhibit partial defects in gut granule biogenesis and are thus sensitized to reveal gene function in the pathway to investigate whether SAND-1/Mon1 and RAB-7 play roles in trafficking to gut granules not apparent in an otherwise wild-type background. We found that mutations in sand-1 did not alter the Glo phenotype of apt-7 mutant embryos or adults (Tables 1 and 2). Moreover, the codistribution of gut granule proteins was not significantly different between apt-7 single and apt-7; sand-1 double mutants (Supplemental Figure S4). Thus we find no evidence supporting a role for SAND-1 in gut granule formation. The number of autofluorescent gut granules in AP-3; rab-7 mutant embryos and adults was not obviously different from that in AP-3 mutants (Tables 1 and 2). Similarly, the few autofluorescent gut granules in glo-2 mutant adults and birefringent gut granules in glo-3 mutant embryos remained in double mutants with rab-7 (Tables 1 and 2). However, the introduction the rab-7 mutation weakly enhanced the Glo phenotypes of pgp-2 mutant adults and embryos and glo-3 mutant adults. Similar to our analysis of protein trafficking, our genetic studies point to RAB-7 selectively and subtly contributing to gut granule formation.

GLO-1(ΔG4) mutants suppress ccz-1(-) and glo-3(-)

Our work shows that CCZ-1 and VPS-C/HOPS promote gut granule formation. In the lysosome biogenesis pathway, CCZ-1 likely functions with SAND-1/Mon1 as a GEF to activate RAB-7, which, through its effector HOPS, mediates protein trafficking by promoting membrane tethering and fusion (Nickerson et al., 2009; Zlatic et al., 2011a; Balderhaar and Ungermann, 2013). We show that rab-7 mutants do not phenocopy ccz-1 mutants, suggesting the possibility that a different Rab protein functions downstream of CCZ-1 in trafficking to the gut granule. Recently HPS-4, one of two mammalian homologues of CCZ-1, was identified as a subunit of the GEF that activates Rab32 and Rab38 (Gerondopoulou et al., 2012). GLO-1, which is known to function in gut granule formation, is the C. elegans Rab GTPase most homologous to these proteins (Hermann et al., 2005).

We used an in vivo genetic approach to analyze whether CCZ-1 might function as an upstream activator of the GLO-1 Rab to promote gut granule formation. If CCZ-1 acts as a GEF to activate GLO-1 in the gut granule biogenesis pathway, then it is possible that ccz-1 mutant adults are Glo due to the GLO-1 Rab being in its inactive GDP-bound form. To examine this possibility, we generated three different point mutations in the G4 domain of GLO-1 that are predicted to weaken the molecular interactions between GLO-1 and guanine nucleotides (Figure 9S). Analogous mutations in Rab7, which is in the same Rab subfamily as GLO-1 (Klopper et al., 2012; Rojas et al., 2012), led to greatly increased rates of spontaneous guanine nucleotide exchange in vitro and the ability to bypass the necessity of its corresponding GEF in vivo (Kucharczyk et al., 2001; McCray et al., 2010; Nordmann et al., 2010; Cabrera and Ungermann, 2013). We collectively refer to the GLO-1(K130E), GLO-1(D132A), and GLO-1(I133F) mutations as GLO-1(AG4) (Figure 9S). The mutations were created in a rescuing GFP-tagged form of GLO-1 (Hermann et al., 2005), which was expressed under the control of the vha-6 intestinal cell–specific promoter (Oka et al., 2001; Pujol et al., 2001). When introduced into a glo-1(1wa437) null mutant background (Hermann et al., 2005), two of the point mutants exhibited rescued activity similar to wild-type GLO-1, whereas GLO-1(K130E) was partially active (Table 3). None of the GLO-1(AG4) mutants exhibited dominant Glo phenotypes when expressed in wild type (unpublished data). The majority of ccz-1 mutant young adults expressing GFP::GLO-1 exhibited a low number of autofluorescent compartments, similar to ccz-1 mutants alone (Figure 9, B and C, and Table 3). In contrast, expression of all of the GLO-1(AG4) mutants significantly increased the number of autofluorescent organelles in ccz-1 mutants, with the majority of GLO-1(D132A)–containing animals resembling wild type (Figure 9, D and E, and Table 3). Consistent with the compartments being gut granules, the GLO-1(D132A) and GLO-1(I133F) constructs, which exhibited the strongest suppression, restored acidified organelles in ccz-1 mutants (Figure 9, K–N). These results are consistent with CCZ-1 acting upstream of, or parallel to, the GLO-1 Rab to promote gut granule biogenesis.

We examined the specificity of the genetic interactions between the GLO-1(AG4) and ccz-1 mutants by testing whether the GLO-1(ΔG4) constructs altered the Glo phenotype of other mutants that disrupt gut granule biogenesis. It has been suggested that GLO-4
and its homologues act as a GEF for Rabs similar to GLO-1 (Ma et al., 2004; Hermann et al., 2005). However, the GLO-1(ΔG4) mutants did not suppress the Glo phenotype displayed by glo-4 mutants (Table 3). Similarly, apt-6 mutants were not rescued by introduction of GLO-1(ΔG4) constructs (Table 3), suggesting that the GLO-1(ΔG4) mutants do not generally restore gut granules in mutants with a partial Glo phenotype similar to ccz-1. Finally, we tested the ability of GLO-1(ΔG4) mutants to bypass the activity of BLOC-1, a complex that has evolutionarily conserved roles in LRO biogenesis, and found that the Glo phenotype resulting from mutations in two BLOC-1–encoding subunit genes, glo-2 and snpn-1, were not suppressed by GLO-1(ΔG4) constructs (Table 3).

CCZ-1 and its homologues are believed to function in a heterodimeric complex with a SAND-1/Mon1–related protein (Levine et al., 2013). Our results indicate that SAND-1 does not act with CCZ-1 to promote gut granule biogenesis. To identify other C. elegans genes that could function with CCZ-1, we performed PSI-BLAST searches with SAND-1/HPS1 family members from other species. Starting with Drosophila HPS1 (Lee et al., 2009), the fourth PSI-BLAST iteration led to the identification of C. elegans GLO-3, which shows low but significant similarity to human and Drosophila HPS1 proteins (Figure 9T). The region of highest homology with SAND-1/HPS1 family members contains a Longin domain (Kinch and Grishin, 2006), which consists of five β-strands sandwiched between α-helices (Filippini et al., 2001; Tochio et al., 2001). Longin domains are found in a variety of proteins that mediate membrane trafficking and notably are often present in Rab GEFs, where they mediate interactions with Rabs and other Longin
domain–containing proteins (Collins et al., 2002; Kim et al., 2006; Yu et al., 2009; Marat et al., 2011; Levine et al., 2013; De Franceschi et al., 2014). Predictions of GLO-3 secondary structure suggest that GLO-3 functions similarly to CCZ-1 and are consistent with both proteins acting upstream of the GLO-1 Rab.

**DISCUSSION**

**HOPS function in LRO biogenesis**

We showed that all six proteins predicted to compose the canonical HOPS complex in *C. elegans* are necessary for the formation of autofluorescent gut granules (Figure 1 and Tables 1 and 2). This requirement resembles that seen in *Drosophila melanogaster*, where disrupting the HOPS subunits Vps16A (Pulipparacharuvil et al., 2005), deep orange/Vps18 (Sevioukov et al., 1999), carnation/Vps33A (Akbar et al., 2009), or light/Vps41 (Warner et al., 1998)
leads to altered eye pigment, likely from the reduced number of LROs (pigment granules) present within the retinal epithelium (Lloyd et al., 1998). Similarly, in zebrafish, mutations in \textit{platinum/vps11} (Thomas et al., 2011), \textit{vps18} (Sadler et al., 2005; Maldonado et al., 2006), and \textit{lbk/vps39} (Schonthaler et al., 2008) disrupt LRO biogenesis, reducing the number of epidermal and retinal melanosomes. Only one HOPS subunit, Vps33a, has been shown to facilitate the formation of LROs in mammals; the \textit{buff}/Vps33a partial-loss-of-function mutant exhibits reduced numbers of melanosomes and platelet dense granules in mice (Suzuki et al., 2002; Nguyen and Wei, 2004), both characteristics of HPS (Huizing et al., 2008). Mutations in HOPS subunit encoding genes have not been detected in HPS patients, likely due to their essential roles during mammalian embryogenesis (Messler et al., 2011; Aoyama et al., 2012; Peng et al., 2012).

Paralogues of VPS16 and VPS33 do not appear to function during \textit{C. elegans} gut granule formation (Tables 1 and 2).

### TABLE 3: Effects of GLO-1(ΔG4) mutants on gut granule formation.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Percentage of animals with the specified number of autofluorescent granules in anterior intestinal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Wild type</td>
<td>0</td>
</tr>
<tr>
<td>\textit{apt}-6(ok429)</td>
<td>50</td>
</tr>
<tr>
<td>\textit{apt}-6(ok429) + \textit{gfp::glo-1}</td>
<td>60</td>
</tr>
<tr>
<td>\textit{apt}-6(ok429) + \textit{gfp::glo-1(D132A)}</td>
<td>50</td>
</tr>
<tr>
<td>\textit{apt}-6(ok429) + \textit{gfp::glo-1(I133F)}</td>
<td>20</td>
</tr>
<tr>
<td>\textit{ccz-1}(ok2182)*</td>
<td>19</td>
</tr>
<tr>
<td>\textit{ccz-1}(ok2182) + \textit{gfp::glo-1}</td>
<td>6</td>
</tr>
<tr>
<td>\textit{ccz-1}(ok2182) + \textit{gfp::glo-1(K130E)}</td>
<td>0</td>
</tr>
<tr>
<td>\textit{ccz-1}(ok2182) + \textit{gfp::glo-1(D132A)}</td>
<td>0</td>
</tr>
<tr>
<td>\textit{ccz-1}(ok2182) + \textit{gfp::glo-1(I133F)}</td>
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</tr>
<tr>
<td>\textit{glp-1}(zu437)</td>
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</tr>
<tr>
<td>\textit{glp-1}(zu437) + \textit{gfp::glo-1}</td>
<td>0</td>
</tr>
<tr>
<td>\textit{glp-1}(zu437) + \textit{gfp::glo-1(K130E)}</td>
<td>0</td>
</tr>
<tr>
<td>\textit{glp-1}(zu437) + \textit{gfp::glo-1(D132A)}</td>
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</tr>
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<td>\textit{glo-2}(zu455)</td>
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<td>\textit{glo-2}(zu455) + \textit{gfp::glo-1(D132A)}</td>
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<tr>
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<tr>
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<td>7</td>
</tr>
<tr>
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<tr>
<td>\textit{glo-4}(ok623) + \textit{gfp::glo-1(K130E)}</td>
<td>100</td>
</tr>
<tr>
<td>\textit{glo-4}(ok623) + \textit{gfp::glo-1(D132A)}</td>
<td>100</td>
</tr>
<tr>
<td>\textit{glo-4}(ok623) + \textit{gfp::glo-1(I133F)}</td>
<td>100</td>
</tr>
<tr>
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</tr>
<tr>
<td>\textit{snpn-1}(tm1892) + \textit{gfp::glo-1}</td>
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</tr>
<tr>
<td>\textit{snpn-1}(tm1892) + \textit{gfp::glo-1(D132A)}</td>
<td>59</td>
</tr>
<tr>
<td>\textit{snpn-1}(tm1892) + \textit{gfp::glo-1(I133F)}</td>
<td>63</td>
</tr>
</tbody>
</table>

All strains were grown at 22˚C unless otherwise noted. Individual young adult-stage animals were analyzed using fluorescence microscopy with a rhodamine filter set and were scored for the number of autofluorescent organelles within the anterior intestine located between the pharynx and vulva. The expression of GLO-1 was verified in all animals scored by the presence of GFP. A Fisher’s exact test was used to compare a mutant expressing \textit{gfp::glo-1} to the same mutant expressing a \textit{gfp::glo-1(ΔG4)} variant. \( *p \leq 0.001, *p \leq 0.05, \) lack of asterisk indicates \( p > 0.05. \)

All \textit{ccz-1(-)} animals scored with or without \textit{gfp::glo-1} transgenes were \textit{ccz-1(-)/ccz-1(-)} progeny of \textit{ccz-1(-)/+} adults. They were identified by containing within their uterus embryos with large refractile bodies visible with DIC microscopy.
granule biogenesis in other organisms (Matthews et al., 2005; Akbar et al., 2009). Instead, they act in the formation of different LROs, epidermal lamellar bodies, and platelet α-granules in mammals (Lo et al., 2005; Hershkovitz et al., 2008; Urban et al., 2012) and fibrous body-membranous organelles in C. elegans spermatocytes (Zhu and L’Hernault, 2003; Zhu et al., 2009). It is possible that the canonical HOPS complex (containing VPS11, VPS16a, VPS18, VPS33a, VPS39, VPS41) and an alternate HOPS-related complex (substituting Vps16b for Vps16a and Vps33b for Vps33a) function in the biogenesis of different types of LROs.

Unlike other HOPS-complex mutants in C. elegans, which are maternal effect lethal, the conditional vps-18(tm1125) mutant provided us with the opportunity to assess the function of VPS-18 during endolysosome and LRO biogenesis in the developing intestinal primordium. We found that loss of vps-18 activity resulted in a dramatic increase in LMP-1::GFP–containing conventional endolysosomes (Figure 7), similar to what was seen in coelomocytes of vps-18 mutants (Xiao et al., 2009). Drosophila epithelial cells lacking vps16A or vps33A activity also display increased number of endolysosomes (Pulipparacharuvil et al., 2005; Akbar et al., 2009), and compromising vps39 or vps41 activity in cultured mammalian cells leads to more late endosomes due to their impaired fusion with lysosomes (Pols et al., 2013). Disrupting HOPS-subunit function in C. elegans phagocytes leads to the accumulation of degraded phagosomes, likely due to defects in the fusion of lysosomes with RAB-7–containing phagosomes (Kichen et al., 2008; Xiao et al., 2009). Our data show that LMP-1::GFP compartments in vps-18 mutants more often contained RAB-7 than did those in wild type (Figure 7). This suggests that in the intestinal primordium, VPS-18 promotes the fusion of lysosomes with late endosomes, a well-documented function of the HOPS complex (Epp et al., 2011; Balderhaaar and Ungermann, 2013; Solinger and Spang, 2013). Based on these phenotypes, it is likely that VPS-18 acts as part of the HOPS complex to direct the dynamics of late endosomal and lysosomal membranes in the embryonic intestine.

Our work strongly suggests that VPS-18 mediates gut granule formation due to its activity as a HOPS subunit. Analysis of five distinct gut granule markers indicates that vps-18 mutant embryos completely lack gut granules (Figures 2–5). The reduction in the number of autofluorescent and birefringent gut granules and misaccumulation of birefringent material by mutations or RNAi targeting all of the other HOPS-subunit-encoding genes (Figures 1 and 2 and Tables 1 and 2), including the HOPS-specific genes vps-39 and vps-41, point toward HOPS acting in the biogenesis of gut granules. Furthermore, loss of VPS-18 is predicted to lead to the disassembly of the HOPS complex (Plemel et al., 2011). The only known HOPS-independent function of VPS18 is as a subunit in the related CORVET complex (Nickerson et al., 2009; Balderhaar and Ungermann, 2013; Solinger and Spang, 2013). We did not detect an alteration in gut granule biogenesis when vps-8 activity was disrupted (Tables 1 and 2), which is predicted to specifically disrupt CORVET activity.

The HOPS and AP-3 complexes have evolutionarily conserved roles in protein trafficking to LROs, and their functional interactions are poorly understood (Dell’Angelica, 2009; Zlatic et al., 2011a). In Saccharomyces cerevisiae, which lacks LROs, HOPS mediates the tethering and consumption of Golgi-derived, AP-3–containing vesicles by promoting their fusion with conventional lysosomes (Darsow et al., 2001; Angers and Merz, 2009). This process is mediated by direct physical interactions between VPS41, associated with HOPS on the lysosomal membrane, and subunits of the AP-3 vesicle coat (Darsow et al., 2001; Angers and Merz, 2009; Cabrera et al., 2010). Our double-mutant analysis leaves open the possibility that HOPS functions downstream of AP-3, possibly in the same pathway, during trafficking to gut granules (Table 2 and Figure 8).

Our genetic studies also indicate that HOPS function in gut granule biogenesis extends beyond acting exclusively in the AP-3–trafficking pathway, as vps-18 mutants exhibit a stronger Glo phenotype than when AP-3 activity is abolished, and mutations in both vps-18 and vps-41 modify the Glo phenotype of AP-3 mutants (Tables 1 and 2). There are a number of ways that HOPS might promote gut granule biogenesis independent of AP-3. HOPS functions in the fusion of late endosomes and lysosomes by promoting the assembly and activity of soluble N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) complexes, which mediate membrane fusion (Balderhaar and Ungermann, 2013; Pols et al., 2013; Solinger and Spang, 2013). SNAREs likely mediate trafficking to LROs (Ghiani et al., 2010; Tamura et al., 2011; Yatsu et al., 2013); however, the site of SNARE action and the specific membrane fusion events that they regulate during LRO biogenesis are unknown. HOPS activity has also been implicated in the maturation of early to late endosomes by functioning to inactivate Rab5 on early endosomes, as well as to activate and recruit Rab7 on nascent late endosomes (Wang et al., 2003; Rink et al., 2005; Nordmann et al., 2010; Poteryaev et al., 2010). Organelle maturation, possibly associated with Rab conversion (Hume et al., 2011), occurs during mammalian melanosome biogenesis (Sitaram and Marks, 2012). However, whether organelle maturation is a conserved feature of LRO biogenesis and whether HOPS directly facilitates this process are open questions.

Rab GTPase activity during gut granule formation

In yeast, Rab7 recruits HOPS and its associated tethering and membrane fusion activity (Price et al., 2000; Wurmser et al., 2000; Brett et al., 2008; Plemel et al., 2011). We found that HOPS mutants displayed stronger gut granule protein biogenesis and trafficking defects than did loss of RAB-7 (Figures 1–5), suggesting that HOPS activity in gut granule biogenesis only partially depends on RAB-7 and that HOPS interacts with another Rab or has Rab-independent activities.

Gut granules were formed in rab-7 mutants (Figures 1–3 and Tables 1–2); however, their size was modestly altered (Supplemental Figure S2), and LMP-1 accumulation on nascent gut granules was disrupted (Figures 4 and 5). Similarly, Rab7 activity partially regulates the protein composition of melanosomes (Hida et al., 2011). Rab7 is associated with many different mammalian LROs (Jordens et al., 2006; Kawakami et al., 2008; Zhang et al., 2011; Ambrosio et al., 2012); however, we did not detect RAB-7 on gut granules at the stage when gut granule defects were observed (Figure 6 and Supplemental Figure S2), suggesting that RAB-7 exerts its effects indirectly or at an early point in the trafficking pathway to gut granules. In addition, the different effects of sand-1 and rab-7 mutants on gut granule formation (Figures 4 and 5) point to an LRO-related function of RAB-7 that is independent of SAND-1/Mon1–mediated activation.

C. elegans RAB-7 mediates the trafficking between early and late endosomes (Poteryaev et al., 2007), the fusion between lysosomes and cell corpse–containing phagosomes (Yu et al., 2008; Guo and Wang, 2010), and yolk degradation (Grant and Hirsh, 1999; Britton and Murray, 2004). In these processes, a complex of CCZ-1 and SAND-1 likely functions as a GEF to generate active GTP-bound RAB-7 (Poteryaev et al., 2007, 2010; Kichen and Ravichandran, 2010; Nieto et al., 2010; Nordmann et al., 2010; Gerondopoulos et al., 2012; Yousefian et al., 2013). Given the subtle effects of rab-7 mutants on gut granules, it was surprising that ccz-1 mutants displayed significant defects in protein trafficking to gut granules.
biogenesis was at work during the creation of this LRO (Herrmann et al., 2005, 2012). Data presented here that HOPS and CCZ-1 function in gut granule formation further support this idea. Although we do not know the identity of the organelle that matures into a gut granule, there are precedents for pre-LROs emerging from the trans-Golgi network, early endosomes, or late endosomes (Marks et al., 2013). It is unlikely that gut granule precursors develop from lysosomes, since blocking lysosome biogenesis at the endosomal stage, immediately before the formation of mature lysosomes, does not disrupt gut granule formation (Treusch et al., 2004; Campbell and Fares, 2010). SAND-1 and Rab-7 are required for normal maturation of early to late endosomes in C. elegans (Poteryaev et al., 2007, 2010). Thus the observations that sand-1 mutant embryos contained gut granules and rab-7 mutant embryos had nearly normal gut granules suggest that late endosomes do not significantly contribute to gut granule biogenesis and that early to late endosome maturation is not a major contributor to gut granule biogenesis. Gut granules are accessible to externally applied endocytic tracers (Clokey and Jacobson, 1986; Hermann et al., 2005), supporting the possibility that the pre–gut granule emerges from the early endosome rather than the trans-Golgi network. Possibly the CDF-2::GFP compartments that accumulate in ccz-1 mutants, which lack known gut granule and lysosomal proteins (Figures 2–5), represent a pre-LRO derived from the early endosome. In any case, our genetic studies suggest a model in which CCZ-1 and GLO-3 act as upstream activators of the GLO-1 Rab, which could function in the maturation of the pre–gut granule and recruit HOPS to regulate membrane fusion events between the pre-LRO and other organelles, such as those generated by AP-3. Additional studies are needed to test this idea and define the precise cellular and molecular processes that mediate gut granule formation. This work will not only provide insights into LRO biogenesis, but will also illuminate the key similarities and differences between lysosome and LRO biogenesis.

MATERIALS AND METHODS

Nematode strains and culture
C. elegans strains were grown at 22°C, unless noted, on NGM media seeded with OP50 bacteria as described (Stiernagle, 2006). N2 was used as the wild-type strain. In standard C. elegans nomenclature, gene names are written in lowercase italics and if appropriate followed by a mutant allele placed in parentheses, and proteins are written in all capitals. The following mutant alleles contained within the N2 background were used: apt-6(ok4229), apt-7(tm920), arl-8(tm2504), ccz-1(ok2182), ccz-1(t1219), glo-1(zu437), glo-2(tm592), glo-2(zu455), glo-3(kx90), glo-3(kx94), glo-4(ok623), him-5(e1490), ppg-2(kx48), rab-7(ok511), sand-1(ok1963), sand-1(or552), snpn-1(tm1892), vps-8(ok2912), vps-11(ok1664), vps-16(ok719), vps-16(ok776), vps-18(tm1125), vps-33(ok2494), vps-39(ok2442), vps-39(tm2253), vps-41(ep402), vps-41(ok3433), and vps-45(tm246). The following transgenes were used: ami3[2cdf-2::gfp; unc-119(+)], ami4[2cdf-2::gfp; unc-119(+)], jcpEx2[ced-1::ccz-1::gfp; unc-119(+)], myo-2::GFP, kxeEx148[f11E1.6::mCherry; rol-6(d)], kxeEx247[vha-6::gfp::glo-1; rol-6(d)], kxeEx249[vha-6::gfp::glo-1(K130E); rol-6(d)], kxeEx252[vha-6::gfp::glo-1(D132A); rol-6(d)], kxeEx254[vha-6::gfp::glo-1(D133F); rol-6(d)], pwi50[lmp-1::lmp-1::gfp; unc-119(+)], pwEx31[lmp-1::lmp-1::gfp; unc-119(+)], and qyEx115[ups-18p::gfp; unc-76(+)]. The strains and alleles are described at WormBase (www.wormbase.org).

Genetic manipulations
Using standard genetic approaches, we found that the embryonic Glo phenotype of vps-18(tm1125) was recessively expressed and
maternally and zygotically rescued. Introduction of a vps-18::gfp transgene whose expression is typically silenced in the germline (Kelly et al., 1997) rescued the embryonic Glo phenotype of vps-18(△), suggesting that embryonic expression is sufficient for vps-18(△) function in gut granule biogenesis. The presence of the vps-18(mt1125) deletion was verified by PCR and DNA sequencing. The Glo phenotype of ccz-1(ok2182) and ccz-1(i2129) embryos was recessively expressed and maternally and zygotically rescued. The adult Glo phenotype of these ccz-1 alleles, although recessive, was not maternally rescued. Introduction of a ccz-1::gfp transgene whose expression is controlled by the ced-1 promoter rescued the embryonic Glo phenotype of ccz-1(△), suggesting that embryonic expression is sufficient for ccz-1(△) function in gut granule biogenesis.

Genetic crosses were used to introduce and make homozygous the integrated cad-2::gfp and lmp-1::gfp transgenes in different mutant backgrounds. Owing to linkage of pwsIs50 and sand-1, sand-1(or552) was crossed into a strain containing the extrachromosomal pwEx31[lmp-1::gfp] transgene. The ccz-1(△) and vps-18(△) mutants were made homozygous after crossing into a kEx148[F11E6.1::mCherry; Rol-6△]-containing strain. The mutations being examined for effects on protein trafficking were homozygous except for rab-7(ok511) and some ccz-1(ok2182)-containing strains, which were maintained as heterozygotes. Homozygous rab-7(△) and ccz-1(△) individuals were identified by the presence of large refractile organelles visible by differential interference contrast (DIC) microscopy.

apt(-); him-5(-) males were typically used to generate apt-6(-) and apt-7(-)-containing double mutants. From crosses of apt(-) with vps-18(△), resulting F2 adults that exhibited wild-type autolucence and corresponding F3 Glo embryos were isolated. These F3s were then cloned out to identify lines exhibiting the apt(-) Glo phenotype upon reaching adulthood. In crosses with vps-41(△) and apt-6(△), the apt-6(△) allele was marked by a closely linked dpy-5(△) mutation, and vps-41(△) was marked by a closely linked unc-6(△) mutation. Dpy Unc F2 offspring of the cross were isolated and scored. From crosses of apt(-) with ccz-1(△) and sand-1(△), resulting F2 progeny that exhibited the apt(-) adult Glo phenotype, which lacked early-stage embryos with large refractile organelles indicative of ccz-1(-) and sand-1(-), were isolated. F3 progeny were cloned out, and those producing embryos with refractile organelles were isolated. From crosses of apt(-) and other glo(-) mutants with rab-7(△), resulting F2 adult progeny exhibiting a Glo phenotype were isolated and allowed to self. Resulting F3 animals that lacked rab-7(△) dead embryos were scored. In all cases multiple double-mutant lines were isolated and scored.

RNAi feeding protocol 1 (Kamath et al., 2001), using bacterial clones from the Ahringer RNAi library (available from Source Bioscience, Nottingham, United Kingdom) or, when these were not present for the gene being studied, the Vidal RNAi library (available from Thermo Scientific, Pittsburgh, PA), was used to knock down gene expression. In some cases rrf-3(pk1426) was used (Simmer et al., 2003). Briefly, gravid adults were bleach to isolate embryos, which were placed onto freshly seeded RNAi plates. When these embryos developed to adulthood, they and their resulting embryonic progeny were scored unless otherwise stated.

Generating GLO-1(ΔG4) mutants

The G4 domain of Glo-1 was altered using site-directed mutagenesis with QuikChange II (Agilent Technologies, Santa Clara, CA) and a vha-6p::gfp::glo-1::let-858 3′UTR plasmid as a template (Hermann et al., 2005). Primers p868, 5′CAGCAGATTTCTTGCAAGTAGCGTCGATACAAACA3′, and p869, 5′GTATTGTCGATATCA-CACTCGTTTGCAAGTAGATTGTCG3′, were used to generate Glo-1(K130E).

Primers P870, 5′CTTGCAAAACAGTGCTATCGACAAATAGTGTCGAGCC3′, and P871, 5′CTCAACAGTTATTGTGCA-TAGCACACTTTGTTGCAAAG3′, were used to generate Glo-1(D132A).

Primers P872, 5′CTTGCAAAACAGTGCTATCGACAAATAGTGTCGAGCC3′, and P873, 5′CTCAACAGTTATTGTGCA-TAGCACACTTTGTTGCAAAG3′, were used to generate Glo-1(Δ133F). Underlined residues denote point mutations. For each mutagenized construct, the coding sequence of gfp::glo-1 was sequenced at the Oregon Health & Science University DNA Services Core facility (Portland, OR) to verify that only the desired point mutation had been introduced. The wild-type vha-6p::gfp::glo-1 plasmid and the three glo-1(ΔG4) mutant constructs were individually co-injected at 1 ng/μl with pRF4 [Rol-6△] at 100 ng/μl into glo-1(△zu437). The resulting transmitting lines with similar expression of GFP::GLO-1 were characterized. Each of the extrachromosomal arrays was assessed for restoration of adult autofluorescent compartments and then moved via a genetic cross into a wild-type background. The arrays did not dominantly alter the number of autofluorescent compartments in wild type. The arrays were crossed from wild type into different glo-mutant backgrounds to generate the analyzed strains.

Microscopy

A Zeiss AxioImager.M2 microscope equipped with DIC, polarization, and fluorescence optics was used for all imaging (Zeiss, Thornwood, NY). Images were captured using an AxioCam MRm digital camera controlled with AxioVision 4.8 software. In all cases, optical Z-series through the regions of interest were captured, and, as indicated in the figure legends, individual optical sections or maximum intensity projections are presented.

L4-stage larvae and adults were immobilized on 3% agarose pads for imaging by mounting animals in 10 mM levamisole (Sigma-Aldrich, St. Louis, MO) or 0.1 μm polystyrene nanobeads (Polysciences, Warrington, PA). Autofluorescent gut granules were visualized with Zeiss 38 (GFP, excitation, BP 470/40; emission, BP 525/50), Zeiss 45 (mCherry; excitation, BP 560/40; emission, BP 630/75), or Zeiss 49 (4′,6-diamidino-2-phenylindole [DAPI]; excitation, G 365; emission, 445/50) filters. Acidic compartments within adults were stained with acridine orange (Sigma-Aldrich) as described (Hermann et al., 2005) and visualized with a Zeiss 45 filter. For each strain, a camera exposure time was identified that did not reveal endogenous autofluorescence in unstained animals, which was used to image acridine orange–stained compartments. ccz-1(ok2182)+ animals containing gfp::glo-1 or gfp::glo-1(ΔG4) transgenes were maintained, and resulting homozygous ccz-1(△) adult progeny with the transgene, which were scored, were identified by both the presence of early-stage embryos with large refractile organelles indicative of ccz-1(△) and the intestinal expression of GFP. In all lines, ccz-1(△) animals lacking GFP expression exhibited a Glo phenotype.

Living embryos were mounted on 3% agarose pads in H2O or 1XM9 for imaging. To immobilize living pretzel-stage animals, excess respiring OP50 bacteria were added with the embryos to induce hypoxia. Within embryos, birefringent material was visualized with polarization optics, and autofluorescent compartments were visualized with a Zeiss 49 filter. LMP-1::GFP was visualized with a Zeiss 38 filter, and F11E6.1::mCherry was visualized with a Zeiss 45 filter. The number of LMP-1::GFP–containing compartments was scored using Z-stacks spanning the entire intestine of 1.5-fold-stage embryos fixed with 100% MeOH to prevent the movement of organelles. LMP-1::GFP is enriched on the plasma membrane of
intestinal cells, enabling the identification of the four cells that compose Int2 and Int3 (Leung et al., 1999) and the quantification of the number of LMP-1::GFP compartments contained within these cells. Acidified compartments within embryos were stained with LysoSensor Green (Invitrogen, Grand Island, NY) as described (Levite et al., 2010) and imaged with a Zeiss 38 filter.

Embryos were fixed and stained with LMP-1 (Hadwiger et al., 2010), PGP-2 (Schroeder et al., 2007), Rab-5 (Audhya et al., 2007), and Rab-7 (Chen et al., 2010) antibodies as described (Leung et al., 1999). The specificity of these antisera using the staining protocol was confirmed in prior work. This protocol extracts the autofluorescent material present within embryonic gut granules. GFP fluorescence was used to visualize CDF-2::GFP and LMP-1::GFP in anti-body-stained embryos. Because rab-7(-) causes maternal effect lethality, homozygous rab-7(-) embryos derived from homozygous rab-7(-) hermaphrodites were identified in mixed populations by their altered 1.5-fold body morphology and enlarged PGP-2 or CDF-2::GFP–marked gut granules. For colocalization studies, two-channel Z-stacks containing signals identifying different organelle-associated proteins were acquired and analyzed. For each of the five 1.5-fold-stage embryos in each experiment, 25–35 organelles containing the protein of interest were randomly selected from within the intestine, and each was manually scored for the presence/colocalization of the other marker. If the signals marking the two proteins overlapped, they were scored as colocalizing. Statistical analysis, which is described in the figure legends, was carried out using Excel 2010 (Microsoft, Redmond, WA).

The images presented are representative of the data used to quantify number, size, and colocalization. The figures were prepared using Photoshop CS2 (Adobe, San Jose, CA), and only contrast was adjusted.

Bioinformatics
Default settings were used in PSI-BLAST searches (Altschul et al., 1997). The location of β-strands and α-helices in the Longin domain of HPS1 are from homology-based structural predictions (Kinch and Grishin, 2006). Secondary structure predictions of GLO-3 were generated using JPRED (Cole et al., 2008), PHD (Przybylski and Rost, 2002), and PSIPRED (Jones, 1999) algorithms.

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