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Published in:
Autophagy

DOI:
10.1080/15548627.2018.1458170

Publication date:
2018

Document version
Publisher's PDF, also known as Version of record

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Citation for published version (APA):
Lund, V. K., Madsen, K. L., & Kjærulff, O. (2018). Drosophila Rab2 controls endosome-lysosome fusion and LAMP delivery to late endosomes. Autophagy, 14(9), 1520-1542. https://doi.org/10.1080/15548627.2018.1458170
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To cite this article: Viktor Karlovich Lund, Kenneth Lindegaard Madsen & Ole Kjaerulff (2018) Drosophila Rab2 controls endosome-lysosome fusion and LAMP delivery to late endosomes, Autophagy, 14:9, 1520-1542, DOI: 10.1080/15548627.2018.1458170

To link to this article: https://doi.org/10.1080/15548627.2018.1458170

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Accepted author version posted online: 25 Jun 2018.
Published online: 13 Aug 2018.

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Rab2 controls endosome-lysosome fusion and LAMP delivery to late endosomes

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ABSTRACT
Rab2 is a conserved Rab GTPase with a well-established role in secretory pathway function and phagocytosis. Here, we demonstrate that Drosophila Rab2 is recruited to late endosomal membranes, where it controls the fusion of LAMP-containing biosynthetic carriers and lysosomes to late endosomes. In contrast, the lysosomal GTase Gie/Arl8 is only required for late endosome-lysosome fusion, but not for the delivery of LAMP to the endocytic pathway. We also find that Rab2 is required for the fusion of autophagosomes to the endolysosomal pathway, but not for the biogenesis of lysosome-related organelles. Surprisingly, Rab2 does not rely on HOPS-mediated vesicular fusion for recruitment to late endosomal membranes. Our work suggests that Drosophila Rab2 is a central regulator of the endolysosomal and macroautophagic/autophagic pathways by controlling the major heterotypic fusion processes at the late endosome.

ARTICLE HISTORY
Received 15 December 2016
Revised 14 March 2018
Accepted 21 March 2018

KEYWORDS
Autophagy; Drosophila; HOPS complex; LAMP; late endosomes; lysosomes; membrane trafficking; Rab proteins; Rab2

Introduction
The endolysosomal pathway plays a critical role in the turnover of cellular components and pathogens, downregulation of endocytosed signaling proteins, mobilization of energy and nutrients, and more specialized functions such as antigen presentation, plasma membrane repair and neurite outgrowth [1–7]. The upstream part of the pathway is characterized by endosomal maturation, whereby Rab5-containing early endosomal vesicles gradually mature into Rab7-containing late endosomes (LEs) through Rab conversion [8], mediated by the Rab7 guanine nucleotide exchange factor (GEF) complex Mon1–Ccz1 [9,10]. In contrast, the downstream delivery of material from LEs to lysosomes is thought to occur by transient and permanent fusion between LEs and lysosomes [11]. Simultaneously, autophagosomes containing cellular components destined for recycling fuse both to LEs and directly to lysosomes [12]. These processes generate hybrid endo- and autolysosomal compartments, in which degradation catalyzed by lysosomal enzymes takes place. Following the completion of digestion, endolysosomes condense to reform small lysosomes that function as storage organelles for resident hydrolases [13].

The current understanding of the mechanism underlying LE-lyosome fusion originates from work in baker’s yeast (Saccharomyces cerevisiae) and relies on the highly conserved heteroeximeric homotypic fusion and vacuole protein sorting (HOPS) tethering complex, along with a set of endosomal and lysosomal soluble NSF attachment protein receptors (SNAREs) [14–19]. In yeast, the HOPS complex bridges the membranes of vacuoles (the yeast equivalent of lysosomes) and LEs by binding to the yeast Rab7 ortholog Ypt7 present on both membranes [15,16,20]. The interaction with Ypt7/Rab7 occurs through the accessory subunits Vps39 and Ltv/Vps41 that attach to the class C core subunits Vps11, Vps16, Vps18, and Vps33 comprising the central part of the HOPS complex [20]. The HOPS complex then induces membrane fusion by the assembly of appropriate SNAREs into a trans-SNARE complex [15,21]. This model is largely believed to hold true also in metazoan systems, albeit with the RAB7A effector RILP together with the small lysosomal GTase ARL8B and its effector PLEKHM2/SKIP playing the role of HOPS receptors in mammals [22–25]. Loss of HOPS subunits causes failure of lysosomal delivery of endocytosed and autophagic material in Drosophila and mammals [26–31].

The lysosome contains a large number of hydrolases that catalyze the degradation of endocytosed material, as well as proteins that mediate supporting functions, ranging from pH-regulating components in the form of the V-ATPase and CLC-7 H+/Cl− exchanger, to metabolite transporters and proteins that control chaperone-mediated autophagy and lysosomal fusion to other organelles [4]. Biosynthetic delivery of lysosomal proteins to the lysosomal system broadly follows two routes. Most soluble lysosomal proteins such as acid hydrolases are transported in coated vesicles from the trans-Golgi network (TGN) to an early stage in the endosomal system, in a pathway that in vertebrates depends on the target protein being labeled with mannose-6-phosphate [32]. In contrast, lysosomal membrane proteins (LMP) such as LAMPS are transported in non-clathrin coated carriers from the TGN directly to a late-stage compartment in the endolysosomal pathway. In mammals, the fusion of these carriers to multivesicular LEs requires VPS41 and the SNARE VAMP7, but not VPS39 or the core HOPS component VPS18 [33]. In Drosophila, transport intermediates containing LMPs fuse to late endosomal compartments in an Ltv/Vps41-dependent manner [29]. In addition, a not insubstantial proportion of LMPs traffic to lysosomes via early endosomes and the plasma membrane [4,29,32–34].
Small GTPases belonging to the Rab family are molecular switches that control organelle identity and properties in all eukaryotic cells [35]. In their active GTP-bound form, Rabs bind to membranes via a lipid anchor and recruit effector proteins such as lipid-modifying enzymes, molecular motors and motor adaptors, and tethering and fusion factors. Transition between the active, membrane-anchored, state of the Rabs and the soluble inactive GDP-bound state is controlled by the GEFs and specific GTPase activating proteins (GAPs).

RAB2 is a highly conserved Rab protein previously linked to Golgi function in both Drosophila and mammals and retrograde trafficking to the endoplasmic reticulum (ER) in mammalian cells [36–38]. More recent data from C. elegans have demonstrated a role in the regulated secretory pathway, in the maturation of dense core vesicles [39–41]. In addition, Rab2 has a role in phagosome maturation [42–45]. In C. elegans, mutations in Rab2/UNC-108 impair progression through the conventional endolysosomal pathway at both early and late stages [46,47]. However, the mechanism behind this function remains poorly understood.

Here we demonstrate that Drosophila Rab2 localizes to membranes of late endosomal and lysosomal compartments and is required for the delivery of LAMP-positive LMP carriers to the endosomal pathway. We also show that Rab2 is indispensable for HOPS-dependent fusion of LEs to lysosomes. In contrast to Rab2, we find that Drosophila Gie/Arl8 is required for LE-lysosome fusion, but not for LMP carrier delivery. While Vps39 is a Rab2 effector [48] and is also involved in LMP carrier delivery, our data suggest that Rab2 does not rely on Vps39 for its role in this process. Surprisingly, we find that recruitment of Rab2 to LE membranes does not require HOPS-mediated vesicular fusion, suggesting that Rab2 is directly recruited from the cytosol during endosomal maturation.

**Results**

**Drosophila Rab2 is essential and ubiquitously expressed**

To examine the function of Rab2 in flies, we generated a P-element imprecise excision allele, Rab2Δ1, in which the entire coding region of Rab2 was deleted, except for the first exon containing the initial 15 codons (Figure 1(a)). Rab2Δ1 homozygous flies survived until the third instar larval stage (L3) and progressively died off during the pupal stages, with ~20% reaching the pharate adult stage, and none eclosing (Figure 1(b)). Hemizygotes carrying Rab2Δ1 over the deficiency Dyf(2R)BSC326 that covers the Rab2 locus showed an essentially identical developmental survival profile, demonstrating that Rab2Δ1 is a null allele (Figure 1(b)). Viability of Rab2Δ1/Dyf(2R)BSC326 hemizygotes was rescued to the wild type and Rab2Δ1/+ heterozygote levels by ubiquitous expression of mCherry-Rab2 under a αTub84B-Gal4 driver (Figure 1(b)).

Because we were unsuccessful in raising a polyclonal antibody against the Rab2 protein, we generated HA-Rab2 and GFP-Rab2 transgenes controlled by endogenous Rab2 regulatory elements (Figure 1(a)) to determine the Rab2 expression pattern and subcellular localization. The HA-Rab2 allele was fully capable of rescuing the viability and fecundity of Rab2Δ1 homozygotes, confirming its functionality (data not shown). When we immunostained rescued L3 larvae for the HA epitope, HA-Rab2 appeared to be ubiquitously expressed, but with strong enrichment in the nervous system and imaginal discs (Figure 1(c)). No significant immunosignal was detected in w1118 controls devoid of HA-Rab2 (Figure 1(c)). Likewise, the GFP-Rab2 transgene showed strong enrichment in larval VNC, and also in the testes (Fig. S1).

**Lysosomal trafficking is perturbed in Rab2 mutants**

Transmembrane lysosomal components such as LAMP and the V-ATPase originating in the ER and Golgi are carried to LEs through a network of intermembrane elements (Figure 1(b–e)) to determine the Rab2 expression levels by immunostaining with an acidophilic dye LysoTracker Red (LTR) (Figure 1(f)). In Rab2Δ1 salivary glands, some GFP-LAMP still reached LTR-labeled lysosomes, but a substantial fraction of the GFP-LAMP signal accumulated in LTR-negative vesicles and vesicular clusters in the cytoplasm (Figure 1(g,h)). In addition, while LTR-positive lysosomal tubules still formed in Rab2Δ1 mutants, the density of LTR-positive structures was...
reduced and the network appeared more fragmented compared to the wild type (Figure 2(a)).

Lt/Vps41 is a HOPS complex subunit required for both LE-lysosome fusion [26,30] and delivery of LMPs to late endosomal and lysosomal compartments [29,33]. Salivary glands from animals hemizygous for light11 (lt11/Df), a strong hypomorphic allele of Drosophila lt/Vps41 [50], showed a similar, but more severe phenotype compared to that of Rab2Δ1 (Figure 2(a,b)). Notably, distinct GFP-LAMP and LTR-positive structures often appeared to cluster with each other in Rab2Δ1 and lt11 mutants, suggesting that loss of Rab2 and Lt/Vps41 impairs fusion rather than clustering (Figure 2(a), brackets).

Autolysosomes accumulate in the fat body tissue of late L3 larvae due to developmentally programmed autophagy [51]. In wild-type late L3 fat bodies, almost all GFP-LAMP localized to large autolysosomes strongly stained with LTR (Figure 2(c)). As in salivary glands, there was a strong decrease in the association between LTR staining and the GFP-LAMP signal in fat bodies of both Rab2Δ1 and lt11/Df animals (Figure 2(c,d)). In Rab2Δ1 and lt11/Df fat bodies, LAMP-positive structures were highly variable in size and GFP intensity, and many showed only weak or no staining with LTR (Figure 2(c)). In addition, the average LTR labeling intensity and size of LTR-positive structures were strongly reduced in lt11/Df compared to controls (Figure 2(c,e)). This is consistent with previous observations that HOPS is necessary for fusion of autophagosomes to endosomes and lysosomes [30,31]. A similar, but somewhat weaker phenotype was observed in the Rab2Δ1 fat body, suggesting that Rab2 is also required for autophagosome delivery to the endolysosomal pathway (Figure 2(c,e)).

Taken together, these results suggest that transport of the transmembrane lysosomal protein LAMP to lysosomes is blocked in Rab2-null mutants.

Rab2 is required for autophagy but not for biogenesis of lysosome-related organelles

During autophagy, autophagosomes fuse to LEs and lysosomes to initiate digestion of their contents. This step can be monitored using a transgene encoding Atg8a fused to tandem GFP-
mCherry (GFP-mCherry-Atg8a). Fusion of the relatively neutral autophagosomes to acidic degradative LEs and lysosomes selectively quenches the more pH-sensitive and proteolytically labile GFP fluorescence, shifting the emitted fluorescence ratio from green/yellow towards red [52]. Autophagy is active in Drosophila photoreceptor cells, as evidenced by massive accumulation of autophagosomes in the eyes of flies with loss-of-function mutations in the HOPS complex components car/Vps33A and dor/Vps18, due to a failure of HOPS-dependent autophagosome-lysosome fusion [28,49]. Consistent with this observation, expression of a GFP-mCherry-Atg8a transgene under an endogenous promoter produced strong red fluorescence in the developing ommatidia in wild-type L3 eye imaginal discs (Figure 2f). When expressed in Rab2Δ1 larvae, the same GFP-mCherry-Atg8a transgene produced a massive accumulation of puncta dispersed along the ommatidial periphery, which labeled strongly for green but weakly for red fluorescence (Figure 2f,g). Together with our previous observation that Rab2Δ1 perturbs the formation of autolysosomes in the late L3 fat body, this strongly suggests that Rab2 is required for autophagosome fusion to organelles of the endosomal and lysosomal pathways in Drosophila.
Formation of lysosome-related organelles (LROs) has many mechanistic commonalities with that of conventional lysosomes, although with some molecular differences [53–55], and also requires the HOPS complex [18,53,56,57]. Mutations in Drosophila HOPS components disrupt the formation of the pigment granule, an insect LRO, leading to a loss of eye coloration [30,58–63]. In view of Rab2’s function in lysosomal trafficking, we investigated if Rab2 is required for pigment granule biogenesis. Surprisingly, eyes of Rab2Δ1 pharate adults did not show any obvious defect in coloration compared to controls (Figure 2(h)). This suggests that Rab2 is not involved in the formation of LROs, but only in the biogenesis of conventional lysosomes.

**Rab2 is necessary for LE-lysosome fusion**

To further elucidate the function of Rab2 in the endolysosomal system, we examined the effect of Rab2 loss on postendocytic trafficking in garland nephrocytes (garland cells), where the endocytic pathway can be readily visualized using an endocytic tracer. These cells form a wreath attached to the junction between the esophagus and proventriculus. Together with the pericardial nephrocytes, they belong to a class of large, highly endocytically active cells that filter and remove toxic materials from the hemolymph [64].

Garland cells take up and deliver endocytic tracer to spherical Rab7-positive LEs within 5 min [65]. Within 40 min of endocytosis, the tracer is delivered to an acidic, LTG-positive lysosomal compartment [66]. We pulsed garland cells from wild-type control and Rab2Δ1 animals for 3 min with Texas Red-avidin (TR-avidin), followed by a 2-h chase. After 2 h, endocytosed TR-avidin resided in a dense LTG-stained tubulo-vesicular network interspersed with larger vacuoles in the cytoplasm of wild-type garland cells (Figure 3(a)). These vacuoles likely correspond to β-vacuoles previously described by other investigators [67–69]. A 7 h chase yielded a similar tracer localization, indicating that this morphology represents the terminal lysosomal compartment in this cell type (Fig. S2). Time-lapse imaging revealed the lysosomal network to be highly dynamic, with tubules constantly extending and retracting and small vesicles shifting position (Video S1). In striking contrast, after a 2 h chase in Rab2Δ1 garland cells all endocytosed avidin was found in large, highly spherical organelles with a complete absence of tubules (Figure 3(b)), see also Figures 4(c), 5(a,c) and 6(a)). This aberrant morphology could be fully rescued by the expression of mCherry-Rab2 in the mutant Rab2Δ1 background (Figure 3(c)).

The lysosomal network in wild-type garland cells also labeled weakly with YFP-Rab7 expressed at a low level (Figure 3(b), middle row, left). In contrast, spherical organelles showing strong YFP-Rab7 labeling of their limiting membrane dominated the cytoplasm of Rab2Δ1 cells (Figure 3(b), middle row, right). The previously described aberrant 2-h tracer-positive compartments corresponded to

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**Figure 3.** Perturbation of postendocytic trafficking in Rab2Δ1 garland cells. Garland cells from L3 larvae pulsed for 3 min with TR-avidin (TR-Av) or A488-avidin (A488-Av), followed by a 2 h chase. Single optical sections through the cortical cytoplasm of living garland cells. (A) Wild-type cells loaded with TR-avidin and stained with LysoTracker Green (LTG). The lysosomal network is detailed in the high-magnification inset, with three tubular extensions marked by arrowheads and a vascular structure by an asterisk. (B) Garland cells from control (left) and Rab2Δ1 (right) animals expressing YFP-Rab7 under direct control of the αTub84BcathD promoter and loaded with TR-avidin. (C) Rab2Δ1 garland cells expressing mCherry-Rab2 and loaded with A488-avidin (live imaging). The inset highlights the tubular network both labeled by endocytic tracer and positive for mCherry-Rab2.
a subpopulation of these organelles (Figure 3(b), right), suggesting that the endocytic pathway is stalled at the late endosomal stage in Rab2\(^{Δ1}\) garland cells. Enlarged Rab7-positive LEs have also recently been reported in mutant garland cells devoid of Vps11, Vps39 and Ltv/Vps41 where LE-lysosome fusion is blocked [69]. An accumulation of enlarged YFP-Rab7-positive LEs was also observed in the larval CNS of Rab2\(^{Δ1}\) animals (Fig. S3A, B) showing that this phenotype is not exclusive to garland cells. Furthermore, GFP-Rab7 over-expressed in somata of large peptidergic neurons in the larval CNS dramatically redistributed from the cytosol to vesicular structures in Rab2\(^{Δ1}\) mutants, compared to the wild type (Fig. S3C, D).

Soluble lysosomal enzymes such as the cathepsins catHD and Cpi/Cath L are delivered to the endosomal pathway from the Golgi apparatus through a Ltv/Vps41-independent, but Dor/Vps18-dependent, pathway [29,33] and are concentrated in lysosomal compartments to undergo proteolytic processing into their mature forms. We performed western blotting of the CNS with attached imaginal disks, pro-ventriculi and garland cells from wild-type, Rab2\(^{Δ1}\) and Ltv/DF L3 larvae, as well as L3 larvae hemizygous for a CRISPR/Cas9-generated deletion mutant of the HOPS subunit Vps39, Vps39\(^{Δ1}\) (see Materials and Methods). We found that although in Rab2\(^{Δ1}\), Vps39\(^{Δ1}/DF\) and Ltv/DF there was a noticeable increase in the amount of immature unprocessed endogenous pro-Cpi/Cath L compared to the wild type, most of the Cpi/Cath L in these mutants existed in the mature, processed form (Figure 4(a), left and middle). This suggests that most soluble lysosomal enzyme still resided in late endosomal or lysosomal compartments. In contrast, in dor\(^{Δ8}\) mutants, in which delivery of lysosomal enzymes to the endolysosomal pathway is blocked [29], most Cpi/Cath L was in the immature pro-form (Figure 4(a), left and middle), and the total amount of Cpi/Cath L was dramatically decreased (Figure 4(a), left and right). This is consistent with soluble lysosomal cargo being secreted to the extracellular space when it cannot be delivered to endosomal compartments [70].

We next examined the localization of soluble lysosomal proteases relative to endocytic cargo, using a catHD-mCherry chimeric reporter [30] in garland cells in combination with live imaging. Due to the high resistance of the mCherry tag to low pH and lysosomal proteolysis, it is highly suitable as a marker for resident lumenal lysosomal proteins [52]. As expected, in wild-type garland cells almost all catHD-mCherry resided in the 2-h tracer-positive lysosomal compartments (Fig. (c,d)). Conversely, in Rab2\(^{Δ1}\) garland cells most of the catHD-mCherry signal resided in small granular structures separate from the spherical tracer-filled late endosomal vacuoles (Figure 4(c,d)). Garland cells hemizygous for Vps39\(^{Δ1}\) displayed a phenotype essentially identical to that of Rab2\(^{Δ1}\) garland cells, both with respect to the localization of catHD-mCherry and the morphological defect of the 2-h tracer-positive compartment (Figure 4(c,d), see also Figure 5(a,c)). In addition, we observed low levels of catHD-mCherry signal in the lumen of the late endosomal vacuoles in both mutants (Figure 4(c), asterisks). Since Vps39 is required for LE-lysosome fusion [26,30,69] and in view of the observation that most of the Cpi/Cath L residing in Rab2\(^{Δ1}\) and Vps39\(^{Δ1}/DF\) tissues was in the processed, mature form (Figure 4(a)), we conclude that the small strongly catHD-positive vesicular compartments in Rab2\(^{Δ1}\) and Vps39\(^{Δ1}\) mutants are storage lysosomes that fail to fuse to tracer-accessible late endosomal vacuoles (see Discussion).

The small GTPase ARLB8 is required for LE-lysosome fusion as the lysosomal HOPS receptor in mammals [25]. Gie/Arl8, the single Drosophila ortholog for mammalian ARLB/A, also localizes to lysosomes [71], but a phenotypic description of Gie/Arl8 loss-of-function in flies is lacking. We found that Gie\(^{e00336}\) and in view of the observation that most of the endogenous Cpi/Cath L was present in the processed mature form in Gie\(^{e00336}/DF\) mutants, with only a very small increase in the immature form compared to the wild type (Figure 4(b)). These results demonstrate that Drosophila Gie/Arl8 is required for LE-lysosome fusion, similar to its mammalian ortholog ARLB8.

We hypothesized that the rate of exchange within the lumen of the organelles of the garland cell terminal lysosomal network is high to ensure efficient mixing of endocytic cargo, resident lysosomal degradative enzymes and accessory proteins. To test this idea, we examined the fate of 2 temporally separated pulses of endocytic tracer. Garland cells were first pulsed with TR-avidin followed by a 2 h chase to allow it to reach the terminal endocytic compartment, and then pulsed with Alexa Fluor 488-avidin followed by an additional 2 h chase. In wild-type cells, this protocol resulted in tracer from the 2 pulses being highly equilibrated in the terminal lysosomal compartment (Figure 5(a,b)). In contrast, in cells from both Rab2\(^{Δ1}\), Vps39\(^{Δ1}/DF\) and Gie\(^{e00336}/DF\) larvae the tracers from the 2 pulses were poorly mixed, with many of the spherical terminal compartments containing a large amount of tracer from one pulse, but little or nothing from the other (Figure 5(a,b)). Similar results were obtained in Ltv/DF cells (Fig. S4A). These data demonstrate that the blocked access to the terminal lysosomal compartment in HOPS, Rab2 and Gie/Arl8 mutant garland cells leads to poor content mixing in the endolysosomal pathway.

We then examined the location of tracer in relation to acidic compartments, labeled by LTG. As described above, and consistent with previous work [66], TR-avidin showed a very high degree of colocalization with LTG in the terminal lysosomal network after a 2 h chase in wild-type garland cells (Figure 5(c,d)). In Rab2\(^{Δ1}\), Vps39\(^{Δ1}/DF\), Ltv/DF and Gie\(^{e00336}/DF\) cells, however, no lysosomal network was apparent, and spherical compartments strongly labeled with LTG filled the cytoplasm (Figure 5(c); Fig. S4B). The overall correlation between LTG and 2-h tracer was also dramatically reduced in these mutants (Figure 5(c,d); Fig. S4B). A quantitative
analysis revealed that in large compartments of wild-type cells, LTG and tracer always colocalized (Figure 5(g)). In contrast, although a fraction of LTG-positive vacuoles had received some tracer after 2 h, most (~70%) remained tracer-negative in Rab2Δ1, Vps39Δ1/Df and Gie00336/Df cells (Figure 5(g)). Also, vacuoles that were tracer-positive but LTG-negative occurred in Rab2Δ1 and Vps39Δ1/Df, but not in wild-type cells, and were fewer in Gie00336/Df cells.
We also observed that tracer-positive LTG compartments in Rab2Δ and Vps39Δ/Df exhibited a LTG signal substantially lower than average, indicating that they were less acidic than the tracer-negative compartments (Figure 5f). This reduction in acidity was much less pronounced in Giee00336/Df (Figure 5f). Surprisingly, the overall LTG labeling intensity of vacuoles in all mutants was much higher than in the wild type (Fig. S5), but also exhibited a
much higher within-cell variation, indicating a lack of equilibration between acidic compartments (Figure 5(e)). Taken together, these results suggest that in Rab2, HOPS and Gie/Arl8 mutants LEs are still capable of acidification, but do not fuse with the terminal lysosomal network.

In summary, these findings demonstrate that garland cells lacking Rab2, HOPS components or Gie/Arl8 are deficient in fusing LEs to cathD-positive lysosomes and cannot form the rapidly exchanging terminal lysosomal network that characterizes wild-type garland cells.

**Rab2, but not Gie/Arl8, is required for lysosomal membrane protein delivery to LEs**

Next, we examined the localization of GFP-LAMP in relation to 2-h tracer-positive endocytic compartments. LAMP and other LMPs are transported from the Golgi in specialized carriers that require Ltvps41 to fuse with LEs [29,33]. Due to the high sensitivity of the GFP signal to pH and proteolytic degradation, our live imaging approach favors the visualization of GFP-LAMP that has not reached endolysosomal compartments. In control garland cells, GFP-LAMP was nearly undetectable, but residual signal largely overlapped with the TR-Avidin-labeled lysosomal network (Figure 6(a)). In Rab2Δ11/Δ11, LtΔ11/Df, and Vps39Δ4/Df garland cells, the GFP-LAMP signal was elevated compared to the wild type and accumulated in vesicles and ring-like conformations encircling 2-h tracer-positive and negative enlarged late endosomal vacuoles (Figure 6(a)). While some of the ring-shaped GFP-LAMP accumulations in LtΔ11/Df and Vps39Δ4/Df cells may suggest the presence of GFP-LAMP in the vacuolar membrane, the location and fragmented nature of most of them indicate that they represent vesicular structures docked at the surface of the vacuoles (Figure 6(a), inset in 3rd column). This notion is consistent with the observation that carriers accumulating in Lt/vps41-depleted S2R+ cells are often docked to late endosomal and lysosomal compartments [29]. Weak luminal accumulation of the GFP signal was also observed in some vacuoles in all 3 mutant genotypes (blue arrow in Figure 6(a), 3rd column; and data not shown), likely due to liberation of the GFP-tag from the transmembrane domain by the action of residual cathepsins. This suggests that while the loss of Rab2, Vps39 and Lt/vps41 strongly impedes the delivery of LAMP carriers to late endosomes in garland cells, it does not completely abolish it.

In contrast to the largely perivacuolar accumulation of GFP-LAMP in Rab2Δ11, LtΔ11/Df, and Vps39Δ4/Df garland cells, almost all the GFP-signal was contained in the lumen of the enlarged tracer-positive and negative late endosomal vacuoles in cells from GieΔ00336/Df animals (Figure 6(a)). To quantify GFP-LAMP delivery to the vacuoles, we measured the ratio between the GFP fluorescence inside 2-h tracer-positive vacuoles and that in a 0.2 µm wide band around them (Figure 6(b)). Although this analysis tends to underestimate the extent of GFP-LAMP delivery – for example because the GFP signal is quenched by intraluminal acidity of Les – it is suitable for revealing relative differences. We found that GieΔ00336/Df cells displayed a significantly higher inside-outside ratio of GFP fluorescence compared to Rab2Δ11, LtΔ11/Df, and Vps39Δ4/Df cells (Figure 6(b)). Furthermore, unlike in Rab2Δ11 and LtΔ11/Df late L3 fat bodies, in which a large proportion of the GFP-LAMP signal did not overlap LTR-positive organelles (Figure 2(c)), in GieΔ00336/Df most of the GFP-LAMP was contained in LTR positive autolysosomes or amphibosomes, similar to the wild type (Fig. S6). The PCC for GFP-LAMP versus LTR in GieΔ00336/Df mutant fat bodies was 0.804 ± 0.019 (mean ± s.e.m; n = 22); this did not differ significantly from the wild-type PCC. These data demonstrate that Lt/vps41, Vps39 and Rab2, but not Gie/Arl8, are required for efficient delivery of LMP carriers to LEs in Drosophila.

**Rab2 does not strongly rely on Vps39 for LAMP delivery to the endolysosomal system**

A striking feature of the Rab2Δ11 phenotype with respect to the GFP-LAMP distribution in garland cells is that much of the GFP-LAMP signal accumulated in larger (up to 500 nm in diameter) brightly fluorescent vesicles (Figure 6(a)). This was not the case in cells from LtΔ11/Df and Vps39Δ4/Df mutants, where GFP-LAMP was predominantly found in smaller vesicular structures often forming tight ring-like clusters around the late endosomal vacuoles (Figure 6(a)). As described above, few GFP-LAMP vesicles of any kind were observed outside of the endosomal vacuoles in GieΔ00336/Df cells (Figure 6(a)). As shown in Figure 6(c), these differences in the GFP-LAMP signal distribution were confirmed using an image processing algorithm counting large particles (diameter >355 nm) of high circularity surrounding the vacuoles (see Materials and Methods).

*Drosophila* Vps39 has recently been reported to be a Rab2 effector [48]. We therefore investigated to what extent the function of Rab2 is mediated by Vps39, by comparing the phenotype of Rab2Δ11; Vps39Δ4 double mutants to that of the Rab2Δ11 and Vps39Δ4/Df single mutants. Surprisingly, the GFP-LAMP delivery phenotype in Rab2Δ11; Vps39Δ4 garland cells was stronger than in Rab2Δ11 and Vps39Δ4/Df cells, with a significantly lower inside-outside ratio of GFP fluorescence for the 2-h tracer-filled vacuoles (Figure 6(b)). In addition, the late endosomal vacuoles tended to be somewhat larger and more irregularly shaped in double-mutant garland cells compared to Rab2Δ11 and Vps39Δ4/Df cells (Figure 6(a)). Interestingly, the large GFP-LAMP vesicles that characterized Rab2Δ11 mutants were replaced by small vesicles in Rab2Δ11; Vps39Δ4/Df cells (Figure 6(a)). This suggests that large vesicles in Rab2Δ11 cells represent either HOPS-dependent homotypic aggregation or fusion of LMP carriers, or HOPS-dependent heterotypic docking or fusion of LMP carriers to a different organelle.

To further establish the nature of the interaction between the Rab2Δ11 and Vps39Δ4 alleles, we examined the GFP-LAMP granule accumulation initially observed in the fixed Rab2Δ11 CNS (Figure 1(d)). As expected, GieΔ00336/Df showed the least amount of granular GFP-LAMP accumulation among all the mutants tested, consistent with GFP-LAMP still being delivered to degradative LEs in the absence of Gie/Arl8A (Figure 7(a,c)). Rab2Δ11 and Vps39Δ4/Df showed similar amounts of GFP-LAMP signal accumulating in
granular structures in the dorsal aspect of L3 brain hemispheres, whereas \( \text{l}t^{11}/\text{Df} \) displayed a slightly lower granular GFP-LAMP signal (Figure 7(a,c)). However, while the shape and distribution of the GFP-LAMP granules were largely identical in \( \text{Vps39}^{31}/\text{Df} \) and \( \text{lt}^{11}/\text{Df} \) brains, the GFP-LAMP distribution in \( \text{Rab2}^{31} \) brains showed several unique features. Most prominent was a massive accumulation of clustered GFP-LAMP granules in a paired structure corresponding to the mushroom body calyx (Figure 7(a)). Strikingly, \( \text{Rab2}^{31}; \text{Vps39}^{31} \) double-mutant brains showed a strong increase in granular GFP-LAMP signal compared to \( \text{Rab2}^{31} \) and \( \text{Vps39}^{31}/\text{Df} \) single mutants (Figure 7(a,c)). Furthermore, the \( \text{Rab2}^{31}; \text{Vps39}^{31} \) brains also exhibited synthetic effects in the form of a marked accumulation of diffuse GFP in the brain surface and the appearance of large GFP-LAMP-positive vacuoles up to 2 \( \mu \text{m} \) in diameter (Figure 7(a)). In addition, western blotting revealed that the relative amount of unprocessed pro-\text{Cp1/Cath} L was elevated even further in \( \text{Rab2}^{31}; \text{Vps39}^{31} \) tissues compared to \( \text{Rab2}^{31} \) and \( \text{Vps39}^{31} \) (Figure 4(b)), again illustrating that the endolysosomal defect is more severe upon loss of both \( \text{Rab2} \) and \( \text{Vps39} \) compared to the loss of either protein.

In summary, these data show that \( \text{Rab2} \) and \( \text{Vps39} \) to a large extent function independently in LMP carrier delivery to LEs.

**Rab2 is required for proteolytic degradation of endocytosed material**

To test if the impaired function of the endolysosomal system in \( \text{Rab2} \) mutants impedes proteolytic degradation of endocytosed material, we used DQ-BSA, an endocytic substrate that becomes...
fluorescent upon proteolytic cleavage [73]. We pulsed garland cells with a 1:1 mix of DQ-Green BSA and Alexa Fluor 647-BSA (A647-BSA) and determined the ratio of DQ-BSA and Alexa Fluor 647 fluorescence. A647-BSA served as a marker of endocytic uptake. After 2 h, wild-type garland cells exhibited a strong DQ-BSA fluorescence, indicating proteolysis of the endocytosed tracer (Figure 8(a)). Strikingly, Rab2Δ1 cells exhibited a 3.6-fold reduction in the DQ-BSA:A647-BSA ratio (Figure 8(a,b)). Furthermore, high-magnification imaging of wild-type cells revealed that, while A647-BSA was contained in the tubulovacular lysosomal system, much of the DQ-BSA signal resided in the cytosol (Figure 8(c)). This most likely reflects that the highly fluorescent BODIPY-labeled free amino acids and oligopeptides generated by DQ-BSA proteolysis are exported from the lysosomal lumen into the cytosol by lysosomal transporters. In contrast, in Rab2Δ1 cells, the DQ-BSA signal was generally contained in the endocytic vacuoles, with very little reaching the cytosol (Figure 8(c)). This could indicate that the proteolytic degradation in Rab2Δ1 is only partial and does not generate a substantial amount of small degradation products, or that export of catabolites from the aberrant late endocytic compartments does not occur. These results indicate that proteolytic degradation of endocytosed material is markedly impaired in cells lacking Rab2, presumably as a combined effect of an uneven acidification of LEs (Figure 5(c–g)) and abolished fusion between LEs and lysosomes that function as storage compartments for degradative enzymes (Figure 4(c,d)).

Rab2 localizes to LEs and lysosomes in a HOPS-independent manner

In light of Rab2’s function in LMP carrier delivery to LEs and LE-lysosome fusion, we sought to determine the localization of the Rab2 protein within the endolysosomal system. Wild-type mCherry-Rab2 accumulated to very high levels in the terminal lysosomal compartment in garland cells (Fig. S7A, see also Figure 3(c)). Curiously, the luminal lysosomal accumulation of mCherry-Rab2 was at least partially activity-dependent, as it was much lower in non-active GDP-locked mCherry-Rab2S20N (Fig. S7A), and was also displayed by HA-Rab2

Figure 7. Accumulation of GFP-LAMP in the CNS of Rab2, HOPS and Gie/Arl8 mutant larvae. (A) Micrographs of right brain hemispheres from fixed L3 larvae ubiquitously expressing GFP-LAMP. The plane of section for the top row (labeled ‘1’) is near the dorsal surface of the hemisphere, while that for the middle row (labeled ‘2’) is about 2 µm deeper in the preparation. The schematic in (B) shows the approximate position of the two optical sections in a live brain, with labels ‘1’ and ‘2’ corresponding to those in (A). (A, middle) A prominent structure (brackets) with clusters of GFP-LAMP granules appeared exclusively in Rab2Δ1 mutants and Rab2Δ1; Vps39Δ1 double mutants. (A, bottom) Areas representative of GFP-LAMP signal accumulation at high resolution. Large GFP-LAMP-positive vacuoles, only occurring in Rab2Δ1; Vps39Δ1 double mutants, are also shown (bottom inset). Single optical slices; medial is right, anterior down. (C) The amount of GFP signal contained in GFP-LAMP granules in the dorsal aspect of the brain hemispheres. The following numbers of larvae (hemispheres) were analyzed (ANOVA followed by Tukey HSD test): 16 w1118, 15 Rab2Δ1, 7 Vps39Δ1/Df, 10 lt57/Df, 9 Rab2Δ1; Vps39Δ1, and 11 Gie00336/Df. P-values in black and blue represent comparisons to w1118 and Rab2Δ1; Vps39Δ1, respectively.
expressed under the endogenous Rab2 promoter (Fig. S7B), suggesting that it is part of the normal life cycle of the Rab2 protein. The lumenal level of mCherry-Rab2 was strongly reduced in a Atg7Δ77 null mutant background, in which macroautophagy is abolished [74] (Fig. S7C). Therefore, macroautophagy appears to be a major mechanism contributing to the intra-lysosomal accumulation of Rab2. However, residual accumulation of mCherry-Rab2 in Atg7Δ77 garland cell lysosomal compartments revealed that other pathways, such as endosomal microautophagy [75], must be involved as well.

Though intriguing, the lumenal accumulation of Rab2 in lysosomes confounds visualization of active, membrane-bound Rab2. To overcome this problem, we used Rab2 transgenes fused to the strongly pH-sensitive super-ecliptic pHluorin (pHl). In agreement with the work of others in Drosophila S2 cells [48], the gain-of-function Rab2 proteins pHluorin-RabQ65L and mCherry-RabQ65L overexpressed in garland cells were strongly recruited to the limiting membranes of enlarged spherical endolysosomal compartments that were positive for 2-h tracer and GFP-LAMP (Figure 9(a,c) and Fig. S7A). Moreover, in L3 salivary glands pHluorin-RabQ65L strongly labeled the limiting membrane of both the tubulovesicular lysosomal network described previously and giant LTR-positive vacuoles (3 to 5 µm in diameter) that generally did not appear in controls (Figure 9(e)).

Importantly, overexpressed wild-type pHluorin-Rab2 also labeled the limiting membranes of many of the tubules and vacuoles of the 2-h tracer-positive lysosomal compartment in garland cells (Figure 9(a), open arrowheads). Labeling of endolysosomal membranes could also be observed with wild-type mCherry-Rab2 overexpressed in the Atg7Δ77 background, where the lumenal mCherry signal was reduced (Fig. S7C). Similar results were obtained using the moderately pH-sensitive GFP-Rab2 expressed under the endogenous Rab2 promoter (Figure 9(b)), showing that endolysosomal membrane labeling is not caused by Gal4-driven overexpression. Weak labeling of some of the tubules and vesicles of the LTR-positive lysosomal network in salivary glands with GFP-Rab2 could also be demonstrated (Figure 9(d)), although this signal was orders of magnitude lower than that from adjacent Rab2-positive Golgi bodies (Figure 9(d)). In agreement with previous
work [48,76–78] GFP-Rab2 strongly concentrated in structures that capped or encircled Golgi bodies in salivary glands (Fig. S8).

To determine at what stage in the endolysosomal pathway Rab2 becomes associated with endosomal and lysosomal organelles, we exposed GFP-Rab2 garland cells to TR-avidin-containing medium and imaged them during endocytosis and further downstream processing of the tracer. We found that, in many cells, GFP-Rab2 was present in the membranes of spherical LEs that were filled by tracer as early as approximately 6 min after the start of the experiment (the earliest time point allowed to be evaluated by our method) (Figure 9(b), top).

We then analyzed the circumstances of Rab2’s recruitment to late endosomes in garland cells, by examining the localization of GFP-Rab2 in different mutant backgrounds. In Gie00336/Df cells, GFP-Rab2 was strongly recruited to the limiting membranes of many of the enlarged late endosomal vacuoles (Figure 10(a)), demonstrating that it does not reach LEs through fusion with lysosomal compartments. It has been suggested that Rab2 arrives to LEs on Golgi-derived vesicles in a HOPS-mediated fusion event and is subsequently quickly released from the membrane [48,79]. Surprisingly, we found that in both Vps39Δ/Δf and lt11/Df garland cells GFP-Rab2 was still present on the membranes of many of the 2-h tracer-positive and negative enlarged late endosomal vacuoles (Figure 10(a)).

Collectively, these data show that Rab2 becomes associated with late endosomal membranes independently of HOPS-
mediated vesicular fusion and remains membrane-bound into the lysosomal stage.

**Discussion**

The endolysosomal pathway is critical for cellular functions ranging from energy metabolism and pathogen neutralization to protein and organellar quality control. Here we demonstrate that Rab2 is required at two distinct steps in the endolysosomal pathway in *Drosophila* (Figure 10(b)). First, Rab2 mediates delivery of LAMP-containing lysosomal membrane protein transport intermediates to LEs. Second, Rab2 is necessary for the fusion of LEs to pre-existing lysosomes. In addition, we observe a strong perturbation of macroautophagy in tissues lacking Rab2, consistent with a requirement of Rab2 in fusion of autophagosomes with LEs and lysosomes.

Many LMPs are carried directly from the Golgi apparatus to the endolysosomal pathway in specialized biosynthetic

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**Figure 10.** Location of Rab2 in HOPS component and Gie/Arl8 mutants and schematic representation of the function of Rab2 in the endolysosomal system. (A) Recruitment of Rab2 to the limiting membrane of LEs is not blocked by the absence of Gie/Arl8 or HOPS complex activity. Garland cells from Vps39^{+1}/Df, lt^{+1}/Df, and Gie^{00036}/Df animals expressing GFP-Rab2 under the endogenous Rab2 promoter, pulsed (3 min) and chased (2 h) with TR-avidin. Insets show GFP-Rab2 labeling the limiting membranes of enlarged late endosomal vacuoles in high magnification. (B) Simplified diagram of the endolysosomal trafficking pathways investigated in this study. Material is passed from early endosomes to LEs, which fuse to lysosomes sometimes exhibiting a tubular morphology. Rab2, Gie/Arl8 and the HOPS complex are necessary for LE-lysosome fusion. Lysosomal membrane proteins are transported directly to LEs from the Golgi apparatus in AP-3 dependent carriers [29]. In *Drosophila*, Rab2 and both Lt/Vps41 [29] and Vps39, but not Gie/Arl8, are necessary for efficient delivery of these carriers. In addition, Rab2 and the HOPS complex are required for the fusion of autophagosomes to LEs and lysosomes [30,31,79,89]. In contrast, the AP-3 and HOPS complexes, but not Rab2, are necessary for trafficking to LROs such as pigment granules [30,58–63].
transport intermediates bypassing early endosomal compartments [29,33,80]. While the structure of these transport carriers is very similar across species (they are non-coated and contain internal membranes), the detailed mechanistic requirements of this pathway vary. In yeast, the carriers fuse directly to the vacuole in a process that requires the entire HOPS complex and Ypt7/Rab7 [81,82], whereas in mammals they fuse to LEs depending only on VPS41 [33]. In fly hemocytes, the pathway is similarly targeted to LEs, and depends on Lt/Vps41, but not Dor/Vps18 [29].

We find that Drosophila Rab2 and Vps39 are also required for delivery of LAMP-containing LMP carriers to LEs. Loss of Rab2 elicits a LAMP trafficking phenotype in multiple tissues that in many ways is similar to that caused by loss of Lt/Vps41 and Vps39. Some differences were, however, observed. In particular, in the absence of Rab2 in garland cells, accumulating LMP carriers appeared to aggregate or fuse together (or possibly to non-degradative storage lysosomes) in a Vps39-dependent manner, thus forming larger granules. In contrast, without Lt/Vps41 or Vps39 small LAMP carriers tended to dock to the surface of LE vacuoles, often forming tightly packed ring-like conformations. We also observed that Rab2 is associated with LE membranes, and this association was not visibly perturbed by absence of Vps39 or Lt/Vps41 (Figure 10(a)). Collectively, these data suggest that Rab2 functions as the late endosomal receptor for LMP carrier docking and fusion.

In contrast to Rab2, we found that the small GTPase Gie/Arl8, while being required for LE-lysosome fusion, is not required for LMP carrier delivery to LEs. In addition to showing that these processes clearly differ mechanistically, this fits well with the predominantly lysosomal localization of Drosophila Gie/Arl8 and its mammalian orthologs [25,71].

Transmembrane components of the vacuolar protein pump are among the proteins transported to LEs in LMP carriers, together with LAMP [29]. Interestingly, perturbation of LMP carrier delivery in Rab2, Vps39 or Lt/Vps41 mutants did not prevent acidification of late endosomal vacuoles in garland cells, possibly reflecting that the block in LMP delivery is incomplete or that substantial amounts of V-ATPase are already present in early endosomes [83]. However, we did observe that vacuoles accessed by endocytic tracer after a 2-h chase were less acidic in Rab2 and Vps39 mutants, both compared to tracer-negative vacuoles, and also to tracer-positive vacuoles in Gie/Arl8 mutants where LMP carriers are still efficiently delivered (Figure 5(f,g)). Assuming that the tracer-accessible vacuoles are on average younger than the tracer-inaccessible ones, this suggests that delivery of LMP carriers accelerates the acidification of LEs.

Even though Vps39 is a Rab2 effector [48], combining the loss of Rab2 with a loss of Vps39 almost doubled the strength of the LAMP-trafficking defect in the CNS and considerably enhanced it in garland cells (Figures 6 and 7). This suggests that Rab2 and Vps39 function independently in LMP carrier delivery. Rab2 must therefore recruit other effectors such as non-Vps39 HOPS components or tethering factors unrelated to HOPS to mediate tethering and fusion of LMP carriers to LEs.

In addition to Rab2’s role in delivery of LAMP carriers to LEs, we also find that Drosophila Rab2 is required for LE-lysosome fusion. It is possible that this requirement is indirect and stems from Rab2-dependent LMP carriers supplying LEs with crucial factors needed for LE-lysosome fusion, such as SNAREs and the vacuolar acidification machinery. However, several arguments can be made against this hypothesis. First, the Rab2Δ; Vps39Δ double-mutant experiments demonstrate that the block in LMP carrier delivery to LEs in Rab2 mutants is not complete. In addition, moderate amounts of LMPs traffic to LEs via the plasma membrane [29]. Therefore, moderate delivery of transmembrane fusion factors would be expected to occur and allow for some degree of LE-lysosome fusion. However, the LE-lysosome fusion defect in Rab2 null garland cells is essentially as severe as in cells lacking Gie/Arl8 and Vps39 (Figures 4 and 5), which are presumably directly required for this process. Moreover, while acidification has indeed been proposed to be important for fusion [84], the enlarged LEs in Rab2 mutants still become highly acidic (Figure 5(c) and Fig. S5), even if this acidification is possibly delayed. Finally, recent work in Drosophila has shown that V-ATPase-dependent acidification is not required for fusion of autophagosomes to LEs and lysosomes and for biosynthetic delivery of LMPs to lysosomes, and therefore presumably also not LE-lysosome fusion, but only for lysosomal degradation [85].

Recent work has shown that after ended hydrolytic digestion, endolysosomes form into small non-acidic storage lysosomes containing inactive lysosomal hydrolases [13]. These storage lysosomes can then fuse to LEs and existing endolysosomes to recycle their content of lysosomal constituents and regenerate lysosomal compartments (see model in Fig. S9A). This provides a likely explanation for the presence of small cathepsin-positive, tracer-inaccessible lysosomes when LE-lysosome fusion is abrogated in Rab2, Vps39 and Gie/Arl8 mutant garland cells (Figure 4(c)). We speculate that as the maternal gene products for these factors are depleted during development, the cathepsins that have already been delivered to the lysosomal system become trapped in small storage lysosomes that can no longer fuse with LEs (Fig. S9B). After this time point, cathepsins delivered to endosomes can no longer reach the lysosomal compartment. Consistent with this, we observe low but discernable levels of luminal cathD-mCherry in many of the enlarged LE vacuoles in Rab2, Vps39 and Gie/Arl8 mutants.

The current model for LE-lysosome fusion in mammals entails the ~30-nm long, seahorse-shaped HOPS complex bridging lysosomal and endosomal membranes by binding the RAB7A effector RILP in the LE membrane and ARLB8 and its effector SKIP on the lysosome [22–25]. The binding is accomplished by the Vps39 and Vps41 subunits that are positioned at each end of the HOPS complex, based on electron tomography of the complex in yeast [20]. However, Gillingham et al. detected no strong interaction between Drosophila Rab7 and Vps39 or the sole fly RILP ortholog [48]. Similarly, activated Drosophila Rab7 does not precipitate Lt/Vps41 [48]. In contrast, activated Rab2 precipitates the entire HOPS complex and binds Vps39 directly [48].
find that both *Drosophila* Rab2 and Gie/Arl8 are required for LE-lysosome fusion. We also observe that Rab2 resides on LE membranes, both in the wild type and in the absence of LE-lysosome fusion in *Gie/Arl8* and HOPS mutants (Figure 10(a)). These results suggest that, in *Drosophila*, the HOPS tethering complex predominantly interacts with Rab2 rather than Rab7 on the LE membrane, while Gie/Arl8 (or its conserved effector SKIP) likely functions as the lysosomal HOPS receptor. However, the observation that the combined loss of Rab2 and Vps39 leads to a slight increase in the severity of the morphological phenotype of the stalled late endosomal compartment in garland cells could imply that Rab2 also interacts with other factors involved in the LE-lysosome transition. In addition, Rab7 is still required for LE-lysosome fusion in *Drosophila* [79,86]. This suggests that Rab7 may assist in the tethering process, perhaps by recruiting the conserved LE-lysosomal adaptor PLEKHM1 (FlyBase Annotation Symbol CG6613) which is strongly precipitated by activated *Drosophila* Rab7 [48] and in mammals facilitates LE-lysosome fusion [87,88].

While this work was in revision, two papers have been published describing the role of Rab2 in LE-lysosome fusion and autophagosome-lysosome fusion, respectively [79,89]. Lőrincz et al. use *Drosophila* garland cells and fat bodies and human cells to essentially show the same requirement for Rab2 in LE-lysosome fusion and autophagy described here [79]. Fujita et al. describe the dependence of autophagic clearance and autophagosome delivery to endosomal and lysosomal compartments on Rab2 in *Drosophila* and mouse embryonic fibroblasts [89]. The picture that emerges from our work and the findings of Lőrincz et al. and Fujita et al. is that Rab2 controls all major heterotypic fusion processes at the LE, i.e. biosynthetic LMP carrier delivery, LE-lysosome fusion and autophagosome-LE fusion (Figure 10(b)). Interestingly, mammalian Rab2A has been found to promote LE exocytosis in cancer cells, suggesting that LE-plasma membrane fusion is also controlled by Rab2 [90]. The role of Rab2 in phagocytosis [42,43] likely parallels its function in the endolysosomal pathway, as the two pathways are mechanistically related. A LE-centric role of Rab2 in the endolysosomal pathway also explains why we do not detect any obvious perturbation of LRO formation in Rab2 null flies. Although the LRO biogenesis pathway in many aspects resembles that of conventional lysosomes, there is evidence suggesting that, at least for some types of LROs, it bypasses the LE compartment [53].

An important question is on which membranes the presence of Rab2 is required in LE-related fusion events, and also how Rab2 arrives on endosomal and lysosomal organelles. Lőrincz et al. argue that Rab2 traffics to LEs on the membranes of Golgi-derived vesicles that fuse with Rab7-positive LEs in a HOPS-dependent manner [79]. Likewise, Fujita et al. suggest that Rab2 is present on the outer membrane of autophagosomes that fuse to Rab7-positive LEs or lysosomes [89]. However, as mentioned, we observe that in garland cells Rab2 is bound to the limiting membrane of LE vacuoles even when HOPS-mediated vesicular fusion was blocked by the absence of Vps39 or Lt/Vps41, and when macroautophagy was abrogated by the absence of Atg7. In fact, in Vps39, Lt/Vps41 and *Gie/Arl8* mutants the association of Rab2 with LE vacuoles appeared more pronounced than in wild-type cells. While this does not exclude that Rab2 is present in the membranes of Golgi-derived vesicles or autophagosomes, it suggests that Rab2 is – at least in some cell types – recruited to LE membranes irrespective of vesicular fusion processes and is likely required on the LE membrane to accept incoming traffic of lysosomes, autophagosomes and biosynthetic Golgi-derived LMP carriers. The failure to easily detect wild-type Rab2 on LE membranes in many cell types [48, 79, 89; and data not shown] may be a consequence of very tight regulation of active membrane-bound Rab2 due to its high fusogenicity illustrated by the enlarged aberrant endolysosomal compartments formed by gain-of-function Rab2 (Figure 9(a)(a,e) and Fig. S7A). Autophagic turnover may assist in keeping Rab2 activity down, forming a feed-back loop that controls formation of endolysosomes and autolysosomes.

**Materials and methods**

**Molecular biology**

To generate the wild-type UAS-mCherry-Rab2, the gain-of-function UAS-mCherry-Rab2<sup>S20N</sup>, and the dominant-negative UAS-mCherry-Rab2<sup>Q65L</sup> transgenes, a pUAST-mCherry plasmid was produced by amplifying the mCherry coding region from pV2-mCherry [91] with primers mCh-3 and mCh-4 (primer oligonucleotides specified in Table 1), and subcloning it into pUAST via the EcoRI-NotI restriction sites. Using oligos Rab2-3 and Rab2-4, the Rab2 coding region was then amplified from pUAST-YFP-Rab2, pUASP-YFP-Rab2<sup>S20N</sup> and pUAST-YFP-Rab2<sup>Q65L</sup> plasmids (a gift from Dr. Hugo J. Bellen, Baylor College of Medicine). The resulting fragments were cloned into NotI-XbaI sites of the pUAST-mCherry plasmid.

The genomic HA-Rab2 transgene was generated by amplifying a 4.1 kb fragment from genomic DNA using oligos Rab2-23 and Rab2-24 and cloning it into the SpeI-NotI restriction sites of the pCasper4 vector. The genomic fragment

| Table 1. Oligonucleotides used in the study. |
|-----------------|-----------------|
| **Oligo** | **Sequence** |
| mCh-3 | GATCGTGAATTCACGGCAAATAATGTGAGCAAGGGCGG |
| mCh-4 | GTACCTCACGGCCGCACCTGTCATCGCTCCATCCGG |
| Rab2-4 | CCTACTCTAGACTAGCATAATCAGGAACATCATAAGGATATCCCATGTTTTAAGCTGACGGGGGT |
| Rab2-3 | GTTCCCGCGGCCGCGGTGGAGGGGGCGGAATGTCCTACGCGTACTTGTTCAAA |
| Rab2-23 | GCGTAACTAGTGGCTCCTACGCGTACTTGTTCAAA |
| Rab2-24 | GTCAGGCGGCCGCAGTTGGAATGATCGCAGGAC |
| Rab2-21 | GCCGATCTCGAGCTAGACTAGCATAATCAGGAACATCATAAGGATATCCCATGTTTTAAGCTGACGGGGGT |
| Rab2-22 | GCCGATCTCGAGCTAGACTAGCATAATCAGGAACATCATAAGGATATCCCATGTTTTAAGCTGACGGGGGT |
extended from the second codon of the \textit{Rab2} gene to approximately 1.0 kb downstream of the \textit{Rab2} transcriptional termination site. A second genomic fragment was then amplified that corresponded to the region stretching from 4.7 kb upstream of the \textit{Rab2} start ATG to the start ATG, using oligos \textit{Rab2}-21 and \textit{Rab2}-22. This fragment was inserted via XhoI-Spel restriction sites into the pCasper4 plasmid carrying the first fragment to complete the genomic \textit{Rab2} transgene. The \textit{Rab2}-22 primer includes a sequence coding for YPYDVPDYA, resulting in insertion of a hemagglutinin tag downstream of the \textit{Rab2} start methionine and an intervening glycine, and followed by a short TSG linker before resumption of the wild-type \textit{Rab2} amino acid sequence.

To generate the UAS-pHluorin-\textit{Rab2} and UAS-pHluorin-\textit{Rab2}^{0.85L} transgenes the mCherry cassette in the UAS-\textit{mCherry-Rab2} and UAS-\textit{mCherry-Rab2}^{0.85L} plasmids, respectively, was exchanged for a super-elliptic pHluorin cassette using the GenScript gene editing service. Likewise, to generate the genomic GFP-\textit{Rab2} transgene the HA coding sequence in the pCasper4-\textit{HA-Rab2} construct was exchanged for an EGFP coding sequence using the GenScript gene editing service.

For CRISPR/Cas9-mediated mutagenesis, gRNA target sequences GGAGTCCAACGACTATTCGC and GAAGTC sequences GGAGTCCAACGACTATTCGC and GAAGTC were inserted into the pHFv-U6.2 vector to obtain the two gRNA constructs targeting the \textit{Vps39} gene. The donor DNA construct for homology-directed repair was generated by inserting ~1 kb homology arms flanking the Cas9 cleavage site into the pH-DsRed-attP vector. All DNA work for CRISPR/Cas9 mediated mutagenesis was performed by GenScript.

\textbf{Fly strains and genetics}

Flies were reared on Nutri-Fly™ Bloomington Formulation medium (Genesee Scientific) at 26°C. For experiments involving \textit{Rab2} mutants, larvae were reared on apple juice plates supplemented with yeast paste.

DNA injections and selection of \textit{P-element} transformants in a \textit{w^{1118}} background was performed by BestGene Inc.. The \textit{Rab2}^{e1118} allele was generated by mobilizing the \textit{P-element} \textit{P(PTT-GA)Rab2}^{CA07465} inserted into the first intron of the \textit{Rab2} gene using the \textit{P[ry]+ t7.2} = \textit{Delta2-3}^{99B} transposase. The \textit{UAS-GFP-Rab2}^\textit{Bloomington} stock no. 42706, 386Y-Gal4 (25410), CyO/\textit{Df(2R)BSC326} (24351), \textit{UAS-mito-HA-GFP} (8443), \textit{UASP-RFP.Golgi} (30908), \textit{tubP-Gal4/TM3B} (5138), \textit{Df(3R)C(7;ry^{026}+TM3,RY^b})} Ser^1 \textit{P[ry} + t7.2} = \textit{Delta2-3}^{99B} (1808), \textit{Df(3R)Exel6178, P[w \textit{+ mC} = XP-U]Exel6178/TM6B, Tb^1} (7657), \textit{t^11/In(2LR)Gla}, \textit{w^{Gla-4}} PPO1^{Kc} (26262), \textit{Df(2L)l45}, PPO1^{Kc/SM1}, \textit{lt^16} (26187), \textit{P{Bac[7]}Gic^{00336}/TM6B, Tb^b} (17846), \textit{Df(3R)BSC222/TM6B, Tb^b} (9699), \textit{dor^1/FM6} (28), and \textit{P(PTT-GA)Rab2}^{CA07465} (50821) lines were obtained from the Bloomington Stock Collection. \textit{Gliotactin-Gal4} was a gift from M. Stern (Rice University). \textit{prospero-Gal4} was a gift from Dr. Helmut Krämer (University of Colorado, Boulder, Colorado). \textit{pm-Cherry-GFP-Atg8a} was a gift from Dr. Tor Erik Rusten (University of Oslo, Oslo, Norway). \textit{c929-Gal4} [93] was kindly provided by Dr. Paul Taghert (Washington University, St. Louis).

The \textit{Vps39}^{A741} knockout line was generated using CRISPR/Cas9-catalyzed homology-directed repair according to the method outlined in [94]. In brief, two gRNA-expressing DNA constructs targeted near each end of the coding sequence of the \textit{Vps39} gene were injected into \textit{yw; nos-Cas9 (II-attP40)} embryos expressing Cas9, together with a donor DNA construct based on the pHD-DsRed-attP plasmid. As a result, most of the coding sequence of \textit{Vps39} (3R:18273942–18272027) was replaced with a \textit{DsRed} transgene, allowing for the expression of only the first 120 amino acids of the \textit{Vps39} protein. This covers only a third of the N-terminal β-propeller domain, most likely resulting in a completely non-functional protein. The DNA injections and selection of transformants were performed by BestGene Inc. The \textit{Vps39}^{A741} allele is homozygous lethal, and it is hemizygous lethal when placed over the \textit{Df(3R)Exel6178} deficiency covering \textit{Vps39}. Both \textit{Vps39}^{A741} homozygotes and \textit{Vps39}^{A741/Df(3R)Exel6178} hemizygotes survive until pupation, but die in pharate adult stage, indicating that \textit{Vps39}^{A741} is a null allele.

To establish the lethal stage of \textit{Rab2} mutants, first instar larvae from overnight laying were deposited on apple juice plates supplemented with yeast (10 animals per plate) and allowed to develop at 26°C. The number of pupae, the most advanced developmental stage reached, and the number of eclosed animals was subsequently scored.

\textbf{Immunohistochemistry and preparation for fluorescent protein imaging of fixed tissue}

For immunohistochemistry and fluorescent protein visualization in fixed tissues, third instar (L3) larval fillets were prepared or isolated L3 brains were dissected out in phosphate-buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH\textsubscript{2}PO\textsubscript{4}, 6.5 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 7.4. Isolated brains were briefly stored in Schneider insect cell medium (Life Technologies, A820) prior to fixation. The tissues were fixed in 3.7% formaldehyde in PBS on ice for 2 h or at room temperature (RT) for 45 min, followed by 6 × 10 min washes in PBX (PBS with 0.3% Triton-X 100 [Sigma-Aldrich, T8787]) at RT. After a 2-h blocking step in blocking buffer (PBX with 5% normal goat serum [Sigma-Aldrich, G9023]) at RT, the tissues were incubated for 24 to 48 h with primary antibodies in blocking buffer at 4°C under gentle agitation. They were then washed 6 × 10 min in PBX, incubated with secondary antibodies in blocking buffer for 2 h at RT, and again washed 6 × 10 min in PBX. Finally, the tissues were washed 2 × 5 min in PBS and mounted in ProLong™ Gold antifade reagent (Life Technologies, P36934).

\textbf{Rabbit anti-HRP (1:250) (P-7899, Lot: 083K4840) and monoclonal rat anti-HA (100 ng/ml) (clone 3F10, REF:
11867423001, Lot: 14553800) were from Sigma. Rabbit anti-
GM130 (D. melanogaster) (1:500) (ab30637, Lot:GR199711-1) was
from Abcam. Mouse anti-Rab7 (D. melanogaster) [95]
(1:200) was from Developmental Studies Hybridoma Bank,
University of Iowa.

Secondary antibodies, Alexa Fluor 488 goat anti-rabbit IgG
(H + L) (A11034, lot: 1423009), Alexa Fluor 647 goat anti-
rabbit IgG (H + L) (A21245, lot: 1558736), Alexa Fluor 647
goose anti-mouse IgG (H + L) (A21236, LOT: 1360398) and
Alexa Fluor 647 goat anti-rat IgG (H + L) (A21247, lot:
1524910) were from Life Technologies and were all used in
a concentration of 1:500.

For imaging of YFP-Rab7 and atuf-GFP-LAMP in fixed
third instar (L3) brains, the tissue was dissected out in PBS or
Schneider insect cell medium (Life Technologies, A820) and
fixed in 3.7% formaldehyde dissolved in PBS for 40' at RT or
2 h on ice. The brains were then washed 6 x 10 min in PBX,
followed by 2 x 5 min in PBS and mounted in antifade
reagent.

**Live imaging**

Salivary glands and eye imaginal disks were dissected out of
late L3 larvae and imaged in PBS in an imaging chamber
constructed from vacuum grease and covered with a coverslip.
For live imaging of peptidergic somata in the dorsal ventral
nerve cord (VNC) of 386Y > GFP-Rab7 animals, fillet dissec-
tions of L3 larvae were performed in modified HL3 solution
(70 mM NaCl, 5 mM KCl, 10 mM NaHCO₃, 20 mM MgCl₂,
5 mM trehalose [Merck KGaA, 108216], 115 mM sucrose
[Sigma-Aldrich, S1888], 0.5 mM EGTA, 5 mM HEPES, pH
7.2). The VNC was imaged directly in the dissection dish
using a LSM710 confocal microscope (Carl Zeiss Microscopy
GmbH, Jena, Germany) equipped with a 40x/1.0 dipping
objective.

**Salivary gland and fat body lysotracker red staining**

Salivary glands and fat body tissue was stained in 1 µM
LysoTracker Red DND-99 (Invitrogen, L7528) in PBS for
10 min at RT. After 2 rinses in PBS, the tissues were mounted
for imaging as described above.

**Garland cell fluorescent tracer uptake and imaging**

Proventriculi with attached garland cells were dissected out
of L3 larvae in PBS. The tissue was pulsed with 80 µg/ml
Texas Red or Alexa Fluor 488-conjugated Avidin (Life
Technologies, A820 and A21370) in Schneider medium
with 5% heat-inactivated fetal bovine serum (FBS) for
3 min at RT. This was followed by 4 rinses in PBS and a
chase of 2 h (or a different duration depending on the
experiment) in Schneider medium with 5% FBS at RT. The
tissue was then mounted for imaging in PBS as described for
salivary glands. For TR-Avidin uptake without chase (0 min
chase time), garland cells were mounted for imaging directly
in 80 µg/ml TR-Avidin dissolved in Schneider medium with
5% FBS. Garland cells that had taken up TR-Avidin were
stained in 1 µM LysoTracker Green DND-26 (Invitrogen,
L7526) after the 2 h chase as described above. For sequential
tracer uptake, garland cells were first pulsed with TR-Avidin
and chased for 2 h, then pulsed with A488-Avidin and
chased for an additional 2 h before being mounted for
imaging. Garland cells were imaged live except for HA
staining experiment shown in Figure 4(c).

**DQ-BSA proteolysis assay**

Garland cells were pulsed for 3 min at RT with a mix of 80 µg/mI
Alexa Fluor 647 BSA (Life Technologies, A34785) and
80 µg/ml DQ Green BSA (Life Technologies, D12050) in
Schneider medium without fetal bovine serum and chased
for 2 h at RT, before being mounted for imaging in PBS as
described. Confocal stacks of garland cell clusters were
acquired using a 10x/0.3 objective.

**Microscopy**

All microscopy was carried out at the Core Facility for
Integrated Microscopy (Department of Biomedical Sciences,
University of Copenhagen). Confocal imaging was performed
on LSM 710 and LSM 780 confocal microscopes (Zeiss), using
the following objectives: Plan-Apochromat 63x/1.4 Oil
DICIII, W Apochromat 421462

**Western blotting**

For western blotting, late L3 larval CNS with imaginal discs,
mouthparts, esophagus, pro-ventriculus and garland cells
attached were dissected out in PBS and stored in
Schneider medium with 5% FBS prior to freezing. Upon
thawing, samples were immediately boiled in loading buffer,
run on SDS-PAGE gels and electroblotted to PVDF
membranes. Each sample consisted of pooled tissues from 5
individuals. Membranes were probed with rabbit anti-Cp1/
CathL/Cathepsin L (1:500; Abcam, ab58991) and mouse
anti-Fas2/Fasciclin II (1:25; Developmental Studies
Hybridoma Bank, 1D4, cell culture supernatant), and then
with HRP-conjugated donkey anti-rabbit (1:5000; Thermo
Fisher scientific, 31458) and HRP-conjugated goat anti-
mouse (1:5000) (Thermo Fisher scientific, 31430). Blots
were developed using Amersham ECL Prime Western
Blotting Detection Reagent (RPN2232) and the resulting
chemiluminescence imaged with an Alpha Innotech
MultiImage Light Cabinet equipped with a CCD camera
(Proteinsimple, San Jose, CA, USA).

**Image processing**

Analysis of confocal images was performed using pre-existing
plugins or custom-designed macros in the Fiji distribution of
the ImageJ software [96], supplemented by calculations in
Excel (Microsoft). Details of the macros are available upon
request. Background subtraction was performed by employing
the Fiji rolling ball plugin or after measuring the signal inten-
sity in an empty area located outside the specimen. To the
largest extent possible, thresholding was done objectively
using a fixed percentile (specified below) of the signal intensity distribution.

**GFP-LAMP in the CNS**
To quantify the GFP-LAMP signal in the larval CNS of controls, Rab2Δ1 mutants, and rescuants, sum projections of image stacks were background-subtracted and smoothed (Gaussian blur, 0.1 µm). The 99th percentile of the pixel intensity distribution was set as threshold, before obtaining the total intensity of suprathreshold GFP-LAMP-positive areas (lower size cutoff 0.25 µm²) using the particle analysis feature in Fiji. Essentially the same procedure was used for the experimental series involving controls, Rab2Δ1, Vps39Δ1/Df, lt11/Df, Rab2Δ1; Vps39Δ1, and Gie00336/Df.

**YFP-Rab7 and GFP-Rab7 in the larval CNS**
To obtain size distributions of YFP-Rab7-positive granules in the CNS of Rab2Δ1 mutants and controls, maximum projections of 3 optical slices from the middle of stacks of third instar VNCs were background-subtracted and smoothed. An intensity threshold was set to vary from 200 to 11,800 with an increment of 200. Each threshold setting was followed by a particle analysis (lower size cutoff 0.1 µm²; circularity 0.8–1.0). The particle size distribution corresponding to the threshold resulting in the largest number of particles was evaluated. To assess the distribution of GFP-Rab7 in peptidergic neurons in the larval VNC, a blinded analysis (genotype unknown to the evaluator) was carried out. GFP-Rab7-positive granules were outlined by placing the Fiji wand tool at local intensity maxima in average projections of substacks (5 slices) close to the ventral surface of the VNC.

**GFP-LAMP in garland cells**
To quantify the internalization of GFP-LAMP in the enlarged endosomal vacuoles of Rab2Δ1, Vps39Δ1/Df, lt11/Df, Rab2Δ1; Vps39Δ1, and Gie00336 garland cells, ROIs corresponding to individual vacuoles were generated using particle analysis (lower size cutoff 1 µm²) on the thresholded red (tracer) channel. After switching to the green channel, the mean GFP-LAMP signal intensity in each vacuolar ROI was divided by the mean intensity in a 0.2 µm wide band surrounding the individual ROIs, yielding the density ratio. After automatic thresholding (95th percentile), particle analysis restricted to perivacuolar bands (width 1 µm) was employed to detect GFP-LAMP-positive vesicles (circularity 0.8 to 1.0, area > 0.1 µm²) surrounding the vacuoles.

**LAMP and LysoTracker red colocalization in salivary glands and fat body**
To quantify the fraction of GFP-LAMP signal located outside LysoTracker Red-positive organelles in salivary glands, ROIs with similar signal intensities across preparations were selected. Following smoothing of the GFP-LAMP channel, both channels were thresholded at the 97.5th percentile. This generally led to inclusion of the vesicular aspect of the tubulo-vacuolar network, while leaving out the tubular system. The total intensity of pixel values exceeding the threshold in the GFP-LAMP channel but not in the LysoTracker Red channel, divided by the total intensity of all pixels with intensities above the GFP-LAMP threshold, was used as readout. Quantification of the intensity and/or size of LysoTracker Red-positive and GFP-LAMP-positive structures was done with particle analysis (lower size cutoff 0.5 µm²). In fat bodies, the Pearson correlation coefficient (PCC) for LAMP versus LysoTracker Red was obtained from a square ROI (225*225 µm²).

**LysoTracker green and TR-avidin in garland cells**
To quantify the LysoTracker Green signal in garland cell vacuoles, individual vacuoles were mapped for intensity measurement using a semi-automated approach (lower size cutoff 0.35 µm²). For both LysoTracker Green and TR-avidin, a threshold equal to 7.5% of the maximal vacuole labeling intensity in the analyzed cell was used to classify each vacuole as either labeled or not.

**PCC calculation in garland cells**
Using a macro capable of processing irregularly shaped ROIs the PCC was calculated for each cell, before averaging over all cells to obtain a single PCC value representing the whole preparation.

**Eye disc autophagy**
To determine the ratio between the GFP and mCherry signals emitted by the GFP-mCherry-Atg8a reporter in eye discs, both channels were background-subtracted before applying particle analysis on the red (mCherry) channel to map areas with pixel intensities exceeding the 95th percentile. For each area, the ratio between the mean intensity in the green (GFP) and red channels was calculated, with the average of this ratio representing one disc.

**Garland cell proteolysis**
Proventriculi with garland cells attached were imaged as stacks in toto at low magnification, and the ratio of the total intensities of the DQ-BSA and A647-BSA channels in background-subtracted sum projections was evaluated.

**Statistics**
Analysis of variance (ANOVA), followed by the Tukey HSD (honest significant difference) test or the Scheffé test, was performed using JMP (SAS Institute, Inc.). Student’s t tests and the Kolmogorov-Smirnov test were performed in Excel. When appropriate, data were transformed to a normal distribution by taking the logarithm or the square root before performing statistical tests [97], or nonparametric tests were used (http://www.vassarstats.net/). The level of statistical significance was set to 5%. Data are presented as mean ± s.e.m unless specified otherwise in the legend.

**Abbreviations**
ANOVA: analysis of variance
AP-3 complex: adaptor protein 3 complex
Atg: autophagy-related
BSA bovine serum albumin
CNS central nervous system
Cp1 cysteine proteinase-1
CRISPR clustered regularly interspaced short palindromic repeats
CV coefficient of variation
Df deficiency
ER endoplasmic reticulum
FBS fetal bovine serum
GAP GTase-activating protein
GDP guanosine-5′-diphosphate
GEF guanine nucleotide exchange factor
GFP green fluorescent protein
Gie GTase indispensable for equal segregation of chromosomes
GTP guanosine-5′-triphosphate
HA hemagglutinin
HOPS homotypic fusion and vacuole protein sorting
HSD honest significant difference
LAMP lysosomal-associated membrane protein
LE late endosomes
LMP lysosomal membrane proteins
LRO lysosome-related organelle
LTG LysoTracker Green
LTR LysoTracker Red
PBS phosphate-buffered saline
PBX PBS with Triton X-100
PLEKHM pleckstrin homology domain-containing family M member
PCC Pearson correlation coefficient
Rab ras-related in brain
RILP Rab-interacting lysosomal protein
SKIP SifA and kinesin-interacting SNARE endosomal and lysosomal soluble NSF attachment protein receptor
TGN trans-Golgi network
TR Texas Red
V-ATPase vacuolar-type proton-translocating ATPase
VAMP vesicle-associated membrane protein
VNC: ventral nerve cord
Vps vacuolar protein sorting
YFP yellow fluorescent protein.

Acknowledgments

We thank the Bloomington Stock Center, the Vienna Drosophila Resource Center, and the following colleagues for fly lines and antibodies: Julie Brill, Suzanne Eaton, Gábor Juhász, Helmut Krämer, Tor Erik Rusten, Paul Taghert, and Benjamin White. We are grateful to Ulrik Gether for helpful discussions and Jacob Balslev Sørensen and Stefan Kins for critical reading of the manuscript. Anders BohlPedersen and the staff at the Core Facility for Integrated Microscopy provided excellent technical assistance. We thank the developers of the ImageJ/Fiji image processing package, and Richard Lowry for maintaining the Vassarstats website for statistical computation.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the The Lundbeck Foundation; The Danish Research Council; Overlæge Johan Boserup og Lise Boserups Legat.

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