Molecular mechanisms of developmentally programmed crinophagy in Drosophila

Tamás Csizmadia,1 Péter Lőrincz,1 Krisztna Hagedűs,1 Szilvia Széplaki,1 Péter Löw,1 and Gábor Juhász1,2

1Department of Anatomy, Cell and Developmental Biology, Eötvös Loránd University, Budapest, Hungary
2Institute of Genetics, Biological Research Centre, Hungarian Academy of Sciences, Szeged, Hungary

At the onset of metamorphosis, Drosophila salivary gland cells undergo a burst of glue granule secretion to attach the forming pupa to a solid surface. Here, we show that excess granules evading exocytosis are degraded via direct fusion with lysosomes, a secretory granule-specific autophagic process known as crinophagy. We find that the tethering complex HOPS (homotypic fusion and protein sorting); the small GTPases Rab2, Rab7, and its effector, PLEKHM1; and a SNAP receptor complex consisting of Syntaxin 13, Snap29, and Vamp7 are all required for the fusion of secretory granules with lysosomes. Proper glue degradation within lysosomes also requires the Uvrag-containing Vps34 lipid kinase complex and the v-ATPase proton pump, whereas Atg genes involved in macroautophagy are dispensable for crinophagy. Our work establishes the molecular mechanism of developmentally programmed crinophagy in Drosophila and paves the way for analyzing this process in metazoans.

Introduction

Autophagy refers to several lysosome-mediated self-degradation pathways that occur within eukaryotic cells. Four main types can be distinguished based on how material reaches lysosomes: macroautophagy, microautophagy, chaperone-mediated autophagy, and crinophagy (Marzella et al., 1981; Weckman et al., 2014). In all cases, degradation is performed by lysosomal hydrolases that are active at an acidic pH, which is achieved by the action of the vacuolar/lysosomal proton pump v-ATPase.

Macroautophagy is the best known among these pathways. It starts with the formation of a phagophore cistern, which sequesters various components of the cytoplasm into double-membrane autophagosomes that deliver their cargo to lysosomal degradation (Feng et al., 2014). Autophagosome formation is achieved by the coordinated action of evolutionarily conserved Atg proteins, which form distinct protein complexes. The fusion of autophagosomes with endosomes and lysosomes requires SNAREs, including the autophagosomal Syntaxin 17 and its binding partners, Snap29 and Vamp8 (Vamp7 in Drosophila), together with the small GTPases Rab2, Rab7, and its effectors, PLEKHM1 and the tethering complex HOPS (homotypic fusion and protein sorting; Kimura et al., 2007; Itakura et al., 2012; Takáts et al., 2013, 2014; Jiang et al., 2014; McEwan et al., 2015; Hagedűs et al., 2016; Fujita et al., 2017; Lőrincz et al., 2017).

During microautophagy, part of the lysosomal membrane invaginates and engulfs a portion of the surrounding cytoplasm, which is then degraded within the lysosome (Mijaljica et al., 2011).

The third pathway is chaperone-mediated autophagy, during which the cytoplasmic chaperone Hsc70 recognizes an exposed KFERQ-like amino acid sequence in proteins to be degraded and transports these into the lysosome through a channel formed by the lysosomal membrane protein Lamp2A (Cuervo and Wong, 2014).

During crinophagy, secretory granules directly fuse with lysosomes to ensure the fast breakdown of excess or obsolete secretory material (Weckman et al., 2014). Crinophagy was discovered 50 yr ago by electron microscopical examination of anterior pituitary gland cells in rats (Smith and Farquhar, 1966). This paper provided ultrastructural evidence that the remaining secretory granules fused with lysosomes when secretory activity was suppressed. Similar tightly regulated secretory granule catabolic processes are part of the normal physiology and development of exocrine, endocrine, and neuroendocrine cells to control the secretory granule pool (Weckman et al., 2014). However, the molecular mechanism of crinophagy is unknown.

The salivary gland of fruit flies changes its function during postembryonic development. The gland produces saliva to help food ingestion and intestinal digestion throughout the larval stages. Later on, ~14 h before puparium formation (~14 h relative to puparium formation [RPF]), the gland starts to synthesize glue proteins. Glue granules are released through a burst of secretory activity at the onset of metamorphosis to attach the pupa to a solid surface. After this wave of glue secretion is over, the remaining cytoplasmic granules may be degraded by...
crinophagy according to an early ultrastructural study of *Drosophila pseudoobscura* (Harrod and Kastritis, 1972). In this work, we characterize crinophagy in the popular animal model *Drosophila melanogaster* and identify the gene products that are required for developmentally programmed glue granule degradation in salivary gland cells.

**Results**

*Glue granules are degraded via crinophagy in Drosophila salivary gland cells at the onset of metamorphosis*

To study glue granule degradation in *Drosophila* late larval and early prepupal salivary gland cells, we established fly stocks that allow the monitoring of this process by fluorescent microscopy. The first stock expresses two previously described glue granule reporters combined (Glue-Red and Glue-GFP), which are both attached to the glue granule protein Sgs3 expressed by the sgs3 promoter (Biyasheva et al., 2001; Costantino et al., 2008). If glue granules fuse with lysosomes, the fluorescence of GFP is quenched in the acidic, degradative milieu. As DsRed is less sensitive to the low pH of lysosomes, granules undergoing lysosomal degradation lose GFP signal but retain DsRed fluorescence. This “GlueFlux” reporter system thus allows the monitoring of glue granule acidification and degradation, similar to the GFP-RFP-Atg8a autophagic flux reporter that is commonly used to follow the lysosomal degradation of autophagosomes (Kimura et al., 2007; Nezis et al., 2010; Nagy et al., 2015).

Glue granule biogenesis starts ~14 h before puparium formation (~14 h RPF; Beckendorf and Kafatos, 1976; Biyasheva et al., 2001; Burgess et al., 2011). The first signs of glue granule degradation were observed as early as in late L3 wandering stage (~6 h RPF), based on the appearance of glue granules that are only positive for DsRed (Figs. 1 A and S1 A). Most of the glue granules remain positive for both DsRed and GFP at this stage, indicating that the majority of these vesicles are intact. The ratio of degrading glue granules readily increased during the next few hours of development, culminating in the complete disappearance of intact granules by 4 h RPF. In double-positive granules, we knocked down VhaSFD in the salivary gland cells, which encodes an essential subunit of the v-ATPase proton pump. As a result, most glue granules remained positive for both GFP and DsRed at the white prepupal stage (0 h RPF; Fig. S1 B), unlike salivary glands in wild-type animals, where only one third of the granules were intact at this stage (Fig. 1 C). Staining glands with Lysotracker red, a dye commonly used for acidic lysosomes, confirmed the acidification defect of glue granules in v-ATPase loss-of-function cells (Fig. S1, C–E).

We generated another reporter line for crinophagy in which Glue-GFP expression is combined with a genomic promoter-driven Cathepsin B (CathB) 3xmCherry transgene expressing a fluorescently tagged lysosomal hydrolase. Only a few small CathB dots were detected at ~6 h RPF, and they rarely overlapped with Glue-GFP granules (Figs. 1 E and S1 F). CathB expression strongly increased by ~2 h, and structures positive for both CathB and Glue-GFP appeared (Figs. 1 F and S1 F). GFP fluorescence in double-positive granules was often fainter than in intact granules, indicating that the quenching of Glue-GFP starts soon after fusion with lysosomes. Granules positive for both Glue-GFP and CathB-3xmCherry were seen in large numbers at the 0 h stage (Figs. 1 G and S1 F). Finally, GFP signal practically disappeared, and the distribution of CathB-3xmCherry resembled that of Glue-Red at 4 h RPF (Figs. 1 H and S1 F).

Our third crinophagy reporter line consisted of the Glue-Red reporter and a GFP marker linked to a transmembrane protein fragment targeted to late endosomes and lysosomes (GFP-Lamp1), in which GFP faces the luminal side and is rapidly quenched in lysosomes (Pulipparacharuvil et al., 2005). During crinophagy, secretory granules directly fuse with lysosomes to give rise to a new degrading organelle called the crinosome (Ahberg et al., 1987). In ~6 h RPF larvae, although GFP-Lamp1-positive dots did not overlap with glue granules, rarely, a ring was seen surrounding Glue-Red granules (Figs. 1 I and S1 G), indicating that fusions occurred. By ~2 h RPF, the majority of glue granules acquired GFP-Lamp1 in the crinosomal membrane (Figs. 1 J and S1 G), as expected based on the other two reporter systems.

**An Uvrag-containing Vps34 kinase complex is necessary for proper glue granule degradation**

Atg genes are required for the capture of insulin-containing secretory granules into autophagosomes, but their role in crinophagy has not been investigated (Riahi et al., 2016). Interestingly, *Atg1, Atg2, Atg3, Atg7, Atg8a, Atg9, Atg14* (the specific subunit of the autophagic Vps34 lipid kinase complex), *Atg16*, and *Atg18a* all turned out to be dispensable for secretory granule degradation based on the GlueFlux reporter when compared with controls (Fig. 2, A–D; Fig. S2, A–H; and Fig. S3 A). In contrast, both *Vps34* and *Uvrag* (the specific subunit of the endocytic Vps34 complex) loss of function impaired crinophagy; the majority of granules remained intact in white prepupal gland cells expressing *Uvrag* RNAi or dominant-negative, kinase-dead *Vps34* (Fig. 2, E and F; and Fig. S3 A). Interestingly, GFP-Lamp1 still formed rings around glue granules at ~2 h, similar to control cells, and GFP fluorescence was retained within Glue-Red–positive granules in Vps34 loss-of-function cells at 1 h RPF, unlike in control cells (Fig. 2, G and H; Fig. S2, I and J; and Fig. S3 B), indicating that granules fuse with lysosomes, but the subsequent degradation of cargo is impaired in these cells. This is in line with previous studies by others and us showing that the Uvrag–Vps34 complex promotes trafficking of lysosomal enzymes and membrane proteins (Brown et al., 1995; Zeng et al., 2006; Juhász et al., 2008; Lőrincz et al., 2014; Takáts et al., 2014).

**HOPS tethering complex subunits are required for glue granule degradation in Drosophila**

Because the molecular mechanism of glue granule degradation was unknown, we tested candidate genes for involvement in crinophagy. Membrane fusion usually requires tethers, small GTPases, and SNARE proteins. We started our functional analysis with the HOPS tethering complex, because we and others have recently identified it as essential for autophagosome clearance in mammalian and *Drosophila* cells (Jiang et al., 2014; Takáts et al., 2014).
In wild-type control animals, only one third of the glue granules retain GFP fluorescence at the white prepupal stage (Fig. 3 A), indicating ongoing glue degradation. In contrast, most glue granules remain intact at the same developmental stage in the absence of HOPS subunits, that is, in animals with salivary gland–specific RNAi knockdown of \textit{Vps16a}, \textit{dor/Vps18}, \textit{car/Vps33a}, and \textit{Vps39}, similar to animals mutant for \textit{light/Vps41} (Fig. 3, B–F; and Fig. S3 A). Because \textit{Vps39} and \textit{light} are HOPS-specific subunits in \textit{Drosophila} and are not found in the related tethering complex CORVET (class C core vacuole/endosome tethering; \Lórinicz et al., 2016), these data indicate that HOPS is required for crinophagy.

We used the GFP-Lamp1 reporter to test whether the lack of HOPS indeed prevents the fusion of glue granules with lysosomes. GFP-Lamp1, which is normally seen as a ring around Glue-Red granules in 2 h glands, failed to form rings and often accumulated as small dots around granules upon salivary gland–specific knockdown of \textit{dor/Vps18} (Fig. 3, G and H; and Fig. S3 B). Similarly, CathB failed to reach Glue-GFP granules in \textit{Vps39} RNAi cells, unlike in controls (Fig. 3, I and J; and Fig. S3 C), in line with a block of fusion.
Ultrastructure of glue granule crinophagy

We sought to confirm our fluorescence microscopy data using ultrastructural analysis. Transmission EM of late wandering stage larval glands revealed lots of intact glue granules as well as granules with loose inner structure, which likely represent crinosomes (Fig. 4 A). In line with this, fusion of multivesicular endosomes with glue granules could be detected (Fig. 4, A and A'). The identity of glue granules (Glue-GFP positive), lysosomes (CathB positive), and crinosomes (double positive) were further confirmed by immuno-EM analysis (Fig. S4).

Salivary gland cells contain acid phosphatase–positive vesicles representing primary lysosomes (Harrod and Kastritis, 1972). We could indeed capture the fusion of acid phosphatase positive lysosomes with glue granules (Fig. 4 B). The granules that evade secretion were becoming increasingly more positive for acid phosphatase in older animals (Fig. 4, C and D). In contrast, enlarged glue granules remained negative for acid phosphatase in animals lacking the HOPS subunit light/Vps41 even at 4 h RPF (Fig. 4 E). These results support our model that glue granules in salivary gland cells are degraded by crinophagy, which requires the HOPS-dependent direct fusion of secretory granules with late endosomes and lysosomes.

The small GTPases Rab2 and Rab7 and the SNAREs Syntaxin 13 (Syx13), Snap29, and Vamp7 are required for glue granule to lysosome fusion

Small GTPases play critical roles in vesicular trafficking processes (Stenmark, 2009). We thus tested the small GTPases Rab2 and Rab7 and its binding partner, PLEKHM1, which are involved in autophagosome–lysosome fusion (McEwan et al., 2015; Hegedüs et al., 2016; Fujita et al., 2017; Lórinicz et al., 2017). Gland-specific knockdown of either Rab2 or Rab7 or Plekhm1 caused a glue granule degradation defect, as most granules remained intact at 0 h RPF, unlike in control cells (Fig. 5, A–D; and Fig. S3 A). Moreover, loss of Rab2, Rab7, or PLEKHM1 resulted in the accumulation of Lamp1-positive lysosomes near glue granules instead of ring formation, as in control cells (Fig. 5, E–H; and Fig. S3 B).

SNARE proteins mediate the majority of membrane fusions within cells, likely including glue granule fusion with lysosomes. We thus tested the role of the SNAREs involved in autophagosome–lysosome fusion (Itakura et al., 2012; Takáts et al., 2013). Syntaxin 17 turned out to be dispensable for glue granule degradation based on the GlueFlux reporter; the GFP signal was readily quenched in Syntaxin 17–null mutant animals, similar to controls (Fig. S5, A and B; and Fig. S3 A). In contrast, the GFP signal persisted in the granules in Snap29 and Vamp7 RNAi cells, respectively (Fig. 6, C and D; and Fig. S3 A). We next analyzed the Drosophila SNAREs that are similar to Syntaxin 17 (Qa SNAREs that have a glutamine in the zero ionic layer of the assembled complex; Table S1). Loss of Syx13 caused a glue granule degradation defect similar to Snap29 and Vamp7 compared with controls (Fig. 6, A and B; and Fig. S3 A). Furthermore, delivery of CathB to Glue-GFP granules was reduced (Fig. 6, E–H; and Fig. S3 C). Lamp1-positive lysosomes accumulated near glue granules in Syx13, Snap29 and Vamp7 RNAi animals, unlike labeling crinosome membranes as in controls (Fig. 6, I–L; and Fig. S3 B).

Indeed, most glue granules retained an intact ultrastructure in Rab2, Rab7, Plekhn1, Syx13, Snap29, and Vamp7 RNAi cells, unlike in controls (Fig. 7). Collectively, these findings suggest that Rab2, Rab7 (and its binding partner, PLEKHM1), and the SNAREs Syx13, Snap29, and Vamp7 are all required for glue granule–lysosome fusion.
Syx13, Snap29, and Vamp7 form a SNA RE complex and may associate with HOPS

Our loss-of-function data raised the possibility that Syx13, Snap29, and Vamp7 function as part of the same SNA RE complex. Syx13 is a Qa SNARE, Snap29 has two SNARE domains (Qb and Qc), and Vamp7 is an R SNARE (having an arginine in the zero ionic layer), which fits well with the rule of SNA RE complex assembly. Indeed, FLAG-tagged Syx13 readily coprecipitated both HA-tagged Snap29 and Vamp7 in cultured Drosophila cells (Fig. 8 A). Interestingly, the amount of Vamp7 bound to beads dramatically increased when all three SNARE proteins were coexpressed, similar to what we have observed previously for the Syx17-containing autophagosomal SNA RE complex (Takáts et al., 2013). This phenomenon suggested that Syx13 and Snap29 together bind more efficiently to Vamp7 than Syx13 does alone. We further tested protein interactions in animal lysates. HA-Syx13 coprecipitated both endogenous Snap29 and endogenous Dor/Vps33a (Fig. 8 B), Vps39 (Fig. 8 C), car/Vps33a (Fig. 8 D), and Vps16a (Fig. 8 E), and in htt/Vps41 mutant animals (Fig. 8 F). [G and H] Glue granule fusion with late endosomes and lysosomes at ~2 h. [G] GFP-Lamp1 is seen as rings (arrowheads) around DsRed-positive glue granules, indicating ongoing crinophagy in control gland cells. [H] In contrast, no rings are seen and small GFP-Lamp1–positive lysosomes often accumulate near Glue-Red granules in dor/Vps18 RNAi cells because a block of fusion. [I and J] Presence of lysosomal cathepsin in glue granules at ~2 h. Glue-GFP granules acquire lysosomal CathB in control cells (I; arrowheads in I), unlike in Vps39 knockdown cells (J). The boxed regions of I and J are shown enlarged in I' and J', respectively. Green and/or magenta channels of merged images are also shown separately as indicated. Bars: (A–E, I, and J) 20 µm; (G and H) 3 µm; (I' and J') 5 µm. Please see Fig. S3 (A–C) for quantification of data.
All of these proteins are membrane bound, because small GTPases of the Rab family have lipid anchors, and Syx13 and Vamp7 have a transmembrane domain, respectively. We could rarely detect the wild-type form of Rab2 in the limiting membrane of glue granules/crinosomes (Fig. S5, C and D), whereas the constitutively active (CA), Q65L mutant form that is thought to promote a GTP-locked state was clearly present in the membrane of most granules/crinosomes (Fig. S5, C and D), whereas Rab2 is known as a Golgi Rab, but its active form was re-localized in Rab2-CA RNAi cells, where loss of HOPS prevents fusion. Interestingly, the distribution of Rab2-CA dramatically changed in HOPS knockdown cells; it exhibited a perinuclear localization and was absent from the membrane of most granules, whereas Rab7 was still recruited to granules on this genetic background (Fig. 9, L and M). These data suggest that Rab7 is recruited to glue granules to promote fusion with Rab2-positive lysosomes, similar to our model of Rab2 and Rab7 action during autophagosome–lysosome fusion (Lörincz et al., 2017).

**Crinophagy is dispensable for glue secretion**

Because lysosomes are also secretory organelles in several cell types (Luzio et al., 2014), we tested whether the fusion of lysosomes with glue granules is necessary for their secretion. Glue-GFP signal was readily detected in the gland lumen in animals with salivary gland–specific loss of Vps39/HOPS, Rab2, or Snap29, unlike in EcR RNAi animals that failed to secrete glue (Fig. S5, E–I), because this event is triggered by ecdysone (Biyasheva et al., 2001; Rousso et al., 2016). Thus, crinophagy does not seem to be required for glue granule exocytosis.

**Discussion**

Our body contains numerous types of secretory cells, including exocrine, endocrine, and neuroendocrine cells, whose main function is to either continuously or temporarily produce, store, and exocytose secretory material into the extracellular space. The amount of the released material is tightly controlled according to the needs of the organism. Thus, usually only part of the secretory granule pool is released, and the remaining vesicles are often degraded via crinophagy (Marzella et al., 2018).

Developmentally programmed crinophagy likely plays important roles during postembryonal development in *Drosophila*. Salivary gland cells produce much more glue than it is necessary to fix the pupa to a solid surface at the onset of metamorphosis. This likely ensures a practically unlimited amount of glue to prevent the pupa from falling down and dying in a wet place. Another possible reason for excess glue production is the storage function of polypliod larval tissues including salivary gland cells. Unused glue that is produced by these cells is recycled via crinophagy, and the building blocks may be re-used later, e.g. to prevent the pupa from falling down and dying in a wet place. An alternative possibility is that Syx13 is recruited to glue granules before fusion.
tethering complexes, and small GTPases that are essential for programmed elimination of obsolete glue granules in *Drosophila* salivary gland cells (Fig. 10). This process requires the direct fusion of these secretory granules with lysosomes and late endosomes. Our data reveal that there is a significant overlap between the factors involved in autophagosome–lysosome fusion and crinophagy. Several known players functioning in autophagosome clearance, namely, the HOPS tethering complex (Jiang et al., 2014; Takáts et al., 2014), its direct binding partner, Rab2 (Gillingham et al., 2014; Fujita et al., 2017; Lőrincz et al., 2017), Rab7 (Kimura et al., 2007; Hegedűs et al., 2016), and its effector, PLEKHM1 (McEwan et al., 2015), are required for both processes. Similarly, the SNA RE proteins Snap29 (Qbc) and Vamp7 (R) also function in both types of vesicle fusions, but they seem to form a functional complex with different Qa SNA REs: Syx17 in case of autophagosome–lysosome fusion (Itakura et al., 2012; Takáts et al., 2013) and Syx13 for glue granule–lysosome fusion, respectively. Thus, Qa SNA REs may specify which organelles can undergo fusion with lysosomes. Interestingly, Syx13 has been identified as a genetic modifier of frontotemporal dementia, possibly acting via promoting autophagic flux (Lu et al., 2013). In contrast, Rab2 and HOPS are also known to promote the proper trafficking of lysosomal hydrolases, and these may function together in protein sorting at the Golgi in addition to lysosomal fusions, which is also supported by our recent identification of direct binding between Rab2 and the HOPS subunit Vps39 (Takáts et al., 2014; Lőrincz et al., 2017). Several of these factors are phosphoinositide effectors, including HOPS and Rab7 via its activator, the Ccz1–Mon1 complex (Stroupe et al., 2006; Cabrera et al., 2014; Hegedűs et al., 2016). It is thus possible that the product of Vps39, phosphatidylinositol

**Figure 5.** Rab2, together with Rab7 and its binding partner, PLEKHM1, is required for glue granule–lysosome fusion. (A–D) Glue granule degradation in white prepupal (0 h) salivary glands. The number of intact glue granules retaining GFP signal greatly increases upon knockdown of Rab2 (B), Rab7 (C), and Plekhm1 (D) compared with control cells (A). [E and H] Glue granule fusion with late endosomes and lysosomes at −2 h. GFP-Lamp1 is seen as rings (arrowheads) around Glue-Red granules, indicating ongoing crinophagy in control cells (E). In contrast, no rings are seen and small GFP-Lamp1 dots often accumulate near Glue-Red granules in Rab2, Rab7, and Plekhm1 RNAi cells [F–H] because of a block of fusion. Green channels are shown separately for A–H. Bars: (A–D) 20 µm; (E–H) 3 µm. Please see Fig. S3 (A and B) for quantification of data.
3-phosphate, also contributes to the regulation of glue granule–lysosome fusion, but loss of Vps34 may be compensated by other classes of phosphatidylinositol 3-kinases or other phosphoinositide species during this process, unlike in the case of lysosomal protein trafficking.

The mechanisms of membrane fusion are highly conserved among metazoans, suggesting that the mammalian orthologues of these Drosophila factors may play similar roles in crinophagy. This process is a major degradation route for insulin-containing secretory granules in β cells, and it is strongly up-regulated upon starvation (Orci et al., 1984; Uchizono et al., 2007; Goginashvili et al., 2015). The continuous synthesis, release, and degradation of these granules ensure a steady pool of vesicles. This balance of synthesis, exocytosis, and breakdown is necessary for β cell homeostasis. Degradation of insulin granules occurs via multiple routes: either by the simple and fast direct fusion of secretory granules with lysosomes (crinophagy) or by the capturing of these small granules into autophagosomes (macroautophagy) or lysosomes (microautophagy; Marsh et al., 2007; Weckman et al., 2014). It has been shown that Rab3A−/− β cells are defective in exocytosis, which leads to enhanced secretory granule degradation mainly through crinophagy (Marsh et al., 2007). Crinophagy thus likely plays an important role in controlling insulin secretion.

Crinophagy also occurs in the exocrine pancreas, but its relevance has not been functionally tested because of a lack
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...of insight into its molecular mechanism. Interestingly, one of the major causes of pancreatitis is the premature, intracellular activation of normally secreted digestive hydrolase precursors, including trypsinogen, by lysosomal proteases within a hybrid organelle (Logsdon and Ji, 2013). This triggers the death of acinar cells, leading to inflammation and digestive enzyme insufficiency. Crinophagy is a possible route that may give rise to such hybrid organelles via direct fusion of secretory vesicles with...
lysosomes. Consistently, morphological analysis suggested that ethionine treatment induces necrosis of pancreatic acinar cells because of a blockade of exocytosis followed by premature intracellular activation of digestive hydrolase precursors at least in part via crinophagy (Koike et al., 1982).

Similarly, inhibition of exocytosis in liver cells by vinblastine treatment up-regulates crinophagy (Ahlberg et al., 1987). Isolation and subsequent biochemical characterization of crinosomes confirmed that these organelles contain both secretory material and active lysosomal hydrolases (Glaumann et al., 1989).

These descriptive studies of the liver and the exocrine and endocrine pancreas clearly indicate that the basal rate of crinophagy strongly increases in response to certain stresses. Activation of crinophagy likely protects the cells and the organism by degrading and recycling unnecessary secretory material. However, it may also be detrimental, for example, because of premature activation of intestinal enzymes within acinar cells of the exocrine pancreas.

Collectively, we demonstrate that HOPS, Rab2, Rab7, PLEKHM1, and the SNAREs Syx13, Snap29, and Vamp7 are required for glue protein degradation by promoting the fusion of Lamp1-positive lysosomes with glue granules, whereas the loss of either the v-ATPase proton pump or the UVRAG-containing Vps34 lipid kinase complex leads to a glue granule degradation defect downstream of fusion caused by lysosomal dysfunction (Fig. 10). Our study thus paves the way for functional analyses of crinophagy in metazoan cells.

Materials and methods

Fly stocks

The following fly stocks were obtained from the Bloomington Drosophila Stock Center: Sgs3 (Glue)-GFP (Biyasheva et al., 2001), da-Gal4, UAS-YFP-Rab2, UAS-YFP-Rab29cs, UAS-YFP-Rab7 (Lörincz et al., 2017), Df(3L)BSC119, and RNAi stocks generated by the Transgenic
PBTX (3 × 15 min at RT), and then incubated in blocking solution in PBS for overnight at 4°C). Samples were extensively washed with PBS then fixed with 4% formaldehyde in PBTX (0.1% Triton X-100) (Takáts et al., 2013). In brief, salivary glands were dissected in ice-cold DAPI. HA-Syx13 was detected essentially as described previously (Lörincz et al., 2014). In brief, dissected salivary glands were fixed with 2% formaldehyde, 2% glutaraldehyde, 3 mM CaCl₂, and 1% sucrose in 0.1 M sodium-cacodylate buffer, pH 7.4, overnight. Subsequently, the buffer was replaced with 0.05 M sodium-acetate buffer (pH 5.0, three 5-min washes). Next, the samples were incubated in Gόmόři’s medium (5 mM sodium-β-glycerophosphate and 4 mM lead nitrate) dissolved in acetate buffer for three 5-min incubations and processed as described for transmission EM. Ultrathin sections were analyzed unstained. Substrate-free medium was used for control experiments.

Acid phosphatase cytochemistry
Acid phosphatase reaction was performed essentially as described previously (Lörincz et al., 2014). In brief, dissected salivary glands were fixed with 2% formaldehyde, 2% glutaraldehyde, 3 mM CaCl₂, and 1% sucrose in 0.1 M sodium-cacodylate, pH 7.4, overnight. Subsequently, the buffer was replaced with 0.05 M sodium-acetate buffer (pH 5.0, three 5-min washes). Next, the samples were incubated in Gόmόři’s medium (5 mM sodium-β-glycerophosphate and 4 mM lead nitrate) dissolved in acetate buffer for three 5-min incubations and processed as described for transmission EM. Ultrathin sections were analyzed unstained. Substrate-free medium was used for control experiments.

Immunogold labeling
Progressive lowering temperature embedding and subsequent immunolabeling were performed as previously described (Lörincz et al., 2014). In brief, salivary glands from Glue-GFP, CathB-3xmCherry animals were dissected in PBS and fixed with 4% formaldehyde, 0.05% glutaraldehyde, and 0.2% tannic acid in phosphate buffer (PB; 0.1 M, pH 7.4) overnight at 4°C. Samples were then washed extensively with PB, and free aldehyde groups were quenched with 50 mM glycine and 50 mM NH₄Cl in PB. Salivary glands were then postfixed in 1% uranyl acetate in 0.05 M maleate buffer (3 h at RT). Samples were then dehydrated in a graded series of ethanol, and embedded into Durcupan (Fluka) according to the manufacturer’s recommendations. 70-nm sections were stained in Reynold’s lead citrate and viewed on a transmission electron microscope (JEM-1011; JEOL) equipped with a digital camera (Morada; Olympus) using iTEM software (Olympus).

Transmission EM
Dissected salivary glands were fixed in 3.2% paraformaldehyde, 0.5% glutaraldehyde, 1% sucrose, and 0.028% CaCl₂ in 0.1 N sodium-cacodylate buffer, pH 7.4, for overnight at 4°C. Samples were then postfixed in 0.5% osmium tetroxide for 1 h and in half-saturated aqueous uranyl acetate for 30 min, dehydrated in a graded series of ethanol, and embedded into Durcupan (Fluka) according to the manufacturer’s recommendations. 70-nm sections were stained in Reynold’s lead citrate and viewed on a transmission electron microscope (JEM-1011; JEOL) equipped with a digital camera (Morada; Olympus) using iTEM software (Olympus).
Cell and animal lysates were spun at 30,130 × g for 30 min; (7) primary antibodies diluted in 5% FCS, 2.5% skimmed milk powder, and 1% BSA in TBS overnight at 4°C; (8) 2% FCS, 1.25% skimmed milk powder, and 1% BSA in TBS for 3 × 5 min; (9) secondary antibodies in 2% FCS, 1.25% skimmed milk powder, and 1% BSA in TBS for 90 min; (10) 3 × 5 min TBS; (11) 1% glutaraldehyde in TBS for 10 min; and (12) extensive wash with bi-distilled water. Ultrathin sections were then stained with uranyl acetate. The following primary and secondary antibodies were used: rat anti-mCherry (1:50; Takáts et al., 2013), chicken anti-GFP (1:50; A10262; Life technologies), 18 nm gold-conjugated goat anti-rat (1:50; 112-215-167; Jackson Immunoresearch Laboratories), and 10 nm gold-conjugated rabbit anti–chicken (1:50; EM.RCHL10; BBI Solutions). Imaging was done as for transmission EM analysis.

Statistics
Fluorescence structures from original, unmodified single focal planes were quantified manually. Three to five cells were randomly selected for counting from pictures of control, RNAi, or mutant salivary glands from three to seven animals. In CathB-3xmCherry Glue-GFP experiments, both GFP- and GFP-negative CathB structures >1 μm in diameter were counted as crinosomes, because especially in the later stages the GFP signal was quenched in most crinosomes. In GFP-Lamp1 experiments, GFP-Lamp1 rings around the Glue-Red granules were counted as double positive. For EM analyses, 8–21 cells from two different animals per genotype were evaluated manually.

We used SPSS17 (IBM) for data analysis. Mann–Whitney U tests were used for comparing two samples, and Kruskal–Wallis tests with post-hoc U test were used for comparing multiple samples, as in all cases, at least one variable showed non-Gaussian data distribution (determined by Kolmogorov–Smirnov test of normality). In the box plots, bars show the data ranging between the upper and lower quartiles; median is indicated as a horizontal black line within the box. Whiskers plot the smallest and largest observations. p-values for the relevant comparisons are shown in the panels.

Cell culture, immunoprecipitation, and Western blots
Syx13 (amino acids 1–259) was amplified from the EST LD27581 using the primers 5′-AGTGTCAAGCCTGGAATACATCCC-3′ and 5′-GATCTTGGCGCGGTAGCTCT-3′ and cloned into pUAST-3xFLAG vector. Transfections and immunoprecipitations were performed in D.mel-2 cells using the plasmids pMT-Gal4, UAS-HA-Vamp7, and UAS-HA-Snap29 (Takáts et al., 2013). For in vivo experiments, UAS-HA-Syx13 was expressed systematically using da-Gal4. 100-mg animals of mixed life stages were collected in 1 ml lysis buffer containing 1% Triton X-100, and homogenized using an Ultra-Turrax. 100-mg animals of mixed life stages were collected in 1 ml lysis buffer containing 1% Triton X-100, and homogenized using an Ultra-Turrax T10 (IKA) with S10N-5G disperser for 2 × 10 s on ice. Both cultured cell and animal lysates were spun at 30,130 × g for 2 × 10 min at 4°C. Immunoprecipitation was performed using anti-HA or anti-FLAG agarose beads (A2095 and A2220, respectively; Sigma-Aldrich) according to the manufacturer’s instructions. Finally, beads were boiled in 30 µl Laemmli buffer, followed by Western blot analysis using mouse anti-FLAG (1:2,000; F1804; Sigma-Aldrich), rat anti-HA (1:2,000; clone 3F10; Roche), rat anti-Snap29 (1:3,000; Takáts et al., 2013), and rabbit anti-Dor (1:1,000; Pulipparacharuvil et al., 2005) primary and alkaline phosphatase–conjugated goat anti–rabbit, rabbit anti–mouse, and rabbit anti–rat (all 1:5,000; A3812, A4312, and A6066, respectively; Sigma-Aldrich). Signal was developed using nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (Sigma-Aldrich). Signal was developed using nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (Sigma-Aldrich) in 100 mM Trizma base, 100 mM NaCl, 5 mM MgCl₂, and 0.05% Tween 20, pH adjusted to 9.5 with HCl. Blots were dried and then scanned on a Perfection 4990 Photo scanner (Epson), followed by processing in Photoshop CS3 Extended.

Online supplemental material
Fig. S1 illustrates the effect of v-ATPase loss on glucose degradation and statistical analysis of developmental crinophagy progression. Fig. S2 shows that core macroautophagy genes are dispensable for the degradation of glue granules. Fig. S3 shows quantification of crinophagy in different genetic backgrounds. Fig. S4 shows the identification of intact glue granules, lysosomes and crinosomes in salivary gland cells using immunogold labeling. Fig. S5 contains additional Syntaxin 17, YFP-Rab2, and glue secretion data. Table S1 shows the effect of Qa SNARE inhibitions on the quenching of Glue-GFP.

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