The class III PI(3)K Vps34 promotes autophagy and endocytosis but not TOR signaling in Drosophila

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D egradation of cytoplasmic components by autophagy requires the class III phosphatidylinositol 3 (PI(3))–kinase Vps34, but the mechanisms by which this kinase and its lipid product PI(3) phosphate (PI(3)P) promote autophagy are unclear. In mammalian cells, Vps34, with the proautophagic tumor suppressors Beclin1/Atg6, Bif-1, and UVRAG, forms a multiprotein complex that initiates autophagosome formation. Distinct Vps34 complexes also regulate endocytic processes that are critical for late-stage autophagosome-lysosome fusion. In contrast, Vps34 may also transduce activating nutrient signals to mammalian target of rapamycin (TOR), a negative regulator of autophagy. To determine potential in vivo functions of Vps34, we generated mutations in the single Drosophila melanogaster Vps34 orthologue, causing cell-autonomous disruption of autophagosome/autolysosome formation in larval fat body cells. Endocytosis is also disrupted in Vps34−−/− animals, but we demonstrate that this does not account for their autophagy defect. Unexpectedly, TOR signaling is unaffected in Vps34 mutants, indicating that Vps34 does not act upstream of TOR in this system. Instead, we show that TOR/Atg1 signaling regulates the starvation-induced recruitment of PI(3)P to nascent autophagosomes. Our results suggest that Vps34 is regulated by TOR-dependent nutrient signals directly at sites of autophagosome formation.

Introduction

Engulfment of cytoplasmic material into specialized double-membrane vesicles known as autophagosomes is the defining feature of a process referred to as macroautophagy or simply autophagy. Subsequent fusion of autophagosomes with the endolysosomal network leads to hydrolytic degradation of the sequestered material. This process provides eukaryotic cells with a mechanism for cytoplasmic renewal by which they can rid themselves of defective organelles and protein complexes (Yorimitsu and Klionsky, 2005). In addition, nonselective autophagy can be induced to high levels by starvation, providing an internal source of nutrients on which cells can survive extended periods of nutrient deprivation. Conversely, under some circumstances autophagy may be used as a killing mechanism, acting as an alternative or augmentation to apoptotic cell death (Neufeld and Baehrecke, 2008). As autophagy has been implicated in several physiological and pathological conditions, including neurodegeneration, tumorigenesis, and aging (Huang and Klionsky, 2007), better understanding of the molecular mechanisms controlling autophagy and identification of pharmacological regulators of this process are important goals.

Wortmannin and 3-methyladenine are well established inhibitors of autophagy. These compounds are broad-spectrum phosphatidylinositol 3 (PI(3))–kinase inhibitors that disrupt autophagy by inhibiting Vps34 (Petiot et al., 2000), the enzymatic component of a multiprotein complex which also includes Vps15, Beclin1/Atg6, UVRAG, and Bif-1 in mammals and Vps15, Atg6, and Atg14 in yeast (Mari and Reggiori, 2007). Localized production of PI(3) phosphate (PI(3)P) by Vps34 can act to recruit proteins containing FYVE and PX domains to specific membrane compartments (Lindmo and Stenmark, 2006). In yeast, this Vps34 complex is critical for recruiting autophagy-related (Atg) proteins to the preautophagosomal structure, the yeast-specific site of autophagosome formation (Suzuki et al., 2007). The role...
of PI(3)P in autophagosome biogenesis is less well understood in higher eukaryotes, and whether it functions at the autophagosomal, the donor, or another membrane has not been determined (Pattinigre et al., 2007).

Vps34 is also required more broadly for several vesicular trafficking processes that may have indirect impacts on autophagy. These include sorting of hydrolytic enzymes to the lysosome/vacuole and early steps in the endocytic pathway (Lindmo and Stenmark, 2006). In mammalian cells, autophagosomes have been shown to fuse with early or late endosomes before fusion with lysosomes, resulting in intermediate structures known as amphisomes (Eskeniline, 2005). Recently, mutations in components of the endosomal sorting complex required for transport (ESCRT) complex, which is required for the transition from early to late (multivesicular) endosomes, have been shown to block autophagy by inhibiting autophagosome-endosome fusion (Nara et al., 2002; Lee et al., 2007; Rusten et al., 2007). Thus, the effect of PI(3)-kinase inhibitors on autophagy may be due, in part, to these more general trafficking functions of Vps34.

Recent work has shown that Vps34 can also function in a nutrient-sensing pathway upstream of the target of rapamycin (TOR) in several mammalian cell lines (Byfield et al., 2005; Nobukuni et al., 2005). Disruption of Vps34 activity with blocking antibodies or siRNA was found to inhibit activation of TOR by insulin, amino acids, and glucose (Byfield et al., 2005; Nobukuni et al., 2005). As TOR signaling inhibits autophagy, these findings are at odds with the conserved role of Vps34 in promoting autophagy under starvation conditions, suggesting that distinct complexes or pools of Vps34 may be subject to different modes of regulation.

In this paper, we address how these multiple potential roles of Vps34 are coordinated to regulate autophagy in an intact organism, the fruit fly Drosophila melanogaster. Our findings suggest that despite a critical role for Vps34 in endocytic uptake and recycling, its primary function in autophagy in vivo is limited to its direct role at the nascent autophagosome. We further show that Vps34 is not required for TOR activity in this system and that starvation results in a TOR/Atg1-dependent recruitment of Vps34 activity to the autophagosomal membrane.

Results

Generation of null and hypomorphic Vps34 mutants

A single representative of each of the three classes of PI(3)-kinase is present in D. melanogaster. The class III PI(3)-kinase orthologue (referred to previously as PI3K_59F and herein as Vps34) shows greatest similarity to human Vps34 (hVps34) in the kinase domain, with an overall identity of 37 and 57% to Vps34 proteins in yeast and human (Linassier et al., 1997). Previous work has shown that D. melanogaster Vps34 displays substrate specificity for PtdIns, with high sensitivity to wortmannin and nonionic detergent as is characteristic of this class (Linassier et al., 1997). Vps34 transcripts are expressed widely during development and in adult flies, with expression in the larval fat body and relative enrichment in neuronal and gut tissues (Chintapalli et al., 2007).

To generate loss-of-function mutations in the Vps34 gene, we established isogenic homozygous-viable stocks of the P element line NP1626 (Hayashi et al., 2002) inserted in the Vps34 5′ untranslated region. Transposase-mediated excision of this element generated several lines that displayed hemizygous larval lethality in trans to a deficiency for this region, Df(2R)bw5. By PCR analysis, we identified an excision line that contains a deletion extending 3,349 bases downstream of the P element insertion site (Fig. 1 A). In this line, Δm22, the entire Vps34 coding region is eliminated, thus resulting in a presumptive null allele. In addition, other lines displaying full or partially penetrant lethality were found to contain partial remnants of the excised P element (Fig. 1 A) and likely represent hypomorphic Vps34 alleles, as in the case of ex32. As expected, Vps34 transcripts were undetectable by RT-PCR in Δm22 mutants and were reduced in ex32 animals (Fig. 1 B). To confirm that these lesions specifically affect the Vps34 locus, we used the GAL4–UAS system to ubiquitously express a wild-type Vps34 transgene in mutant animals, resulting in a complete rescue of lethality (unpublished data).

The activity of Vps34 can be monitored in vivo by examining the intracellular localization of FYVE domain–containing proteins (Gillooly et al., 2000). To assay Vps34 function in mutant cells, we used a transgenic UAS line expressing EGFP fused to two tandem copies of the FYVE domain from the early endosomal protein Hrs (GFP-2xFYVE) (Wucherpfennig et al., 2003). For these experiments, we assayed GFP-2xFYVE distribution in FLP-FRT–generated clones of Vps34 mutant cells, which are marked by the loss of a UAS-driven dsRed marker. This system allows side-by-side comparison of mutant and wild-type cells within the same animal. In wild-type fat body cells, GFP-2xFYVE localized primarily to vesicular structures in the perinuclear region, with additional punctate staining occurring at low levels throughout the cytoplasm and at the cell periphery (Fig. 1, C and D). In contrast, GFP-2xFYVE staining was diffuse in Δm22 mutant cells, with no perinuclear localization and few or no apparent punctate structures (Fig. 1 C). Collectively, these results indicate that the Δm22 deletion specifically eliminates Vps34 expression, resulting in a cell-autonomous loss of function.

As a complement to these mutations in the endogenous Vps34 gene, we also generated UAS-regulated transgenic lines that express D. melanogaster Vps34 containing point mutations in conserved residues of the kinase domain (referred to as Vps34KD). Analogous mutations in yeast and mammalian Vps34 result in a kinase-defective protein with dominant-inhibitory properties (Row et al., 2001). Coexpression of Vps34KD with GFP-2xFYVE in FLP-FRT–generated fat body clones (Britton et al., 2002) resulted in diffuse GFP-2xFYVE localization similar to the deletion mutant (Fig. 1 E), which is consistent with a disruption of Vps34 activity in Vps34KD–expressing cells. Incubation of dissected fat body with the PI(3)K inhibitor 3-methyladenine also caused a similar defect in GFP-2xFYVE localization (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200712051/DC1).

Although Vps34 has been localized to several distinct subcellular compartments in different cell types and experimental systems, its highest levels of activity are typically found in the early endosome, and GFP-2xFYVE is commonly used as an
Starvation-induced formation of Lysotracker-positive structures was severely inhibited in clones of $Vps34$ mutant cells and in cells expressing $Vps34$ KD (Fig. 2, A and B), which is consistent with a requirement for $Vps34$ in autophagy induction or progression. Overexpression of wild-type $Vps34$ was not sufficient to induce Lysotracker-positive structures under fed conditions (Fig. 2 C) but did result in a more robust response to starvation (Fig. 2 D).

To examine earlier stages of autophagy, we determined the localization of the autophagosomal marker GFP-Atg8/LC3 in wild-type and $Vps34$ mutant cells. Starvation-induced accumulation of GFP-Atg8 punctae was strongly curtailed in both $Vps34$ mutant and $Vps34$ KD-expressing cells (Fig. 2, E–G), indicating an essential cell-autonomous role for $Vps34$ in autophagosome biogenesis. Expression of $Vps34$ KD also blocked the starvation-induced accumulation of preautophagosomal membranes labeled with GFP-Atg5 (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200712051/DC1). Disruption of $Vps34$ and autolysosome formation was also evident by ultrastructural analysis of starved $Vps34$ mutant larvae (Fig. 2, H–J). Morphometric analysis revealed that the percent volume of cytoplasm occupied by autophagosomes and autolysosomes was reduced by more than 10-fold in both $Vps34$ mutant and animals overexpressing kinase-dead $Vps34$ in the fat body, relative to controls ($P < 0.0001$; Fig. 2 K).

We previously found that a hypomorphic $P$ element allele of $D. melanogaster$ Atg6/beclin 1 results in a moderate early endosomal marker (Gillooly et al., 2000). To test whether the perinuclear localization of GFP-2xFYVE in the fat body reflects the early endosomal compartment in this cell type, we examined the localization of other early endosomal markers. Both Hrs and Rab5-GFP displayed a similar perinuclear pattern which overlapped with GFP-2xFYVE or myc-2xFYVE and which was disrupted in $\Delta m22$ mutant cells and in cells expressing $Vps34^{kd}$ (Fig. S1). We also found that the GFP-2xFYVE–labeled compartment was readily accessible to the endocytic tracer Texas Red (TR) avidin (see subsequent sections). In contrast, no colocalization was observed between GFP-2xFYVE and the Golgi marker p120 nor the late endosomal/lysosomal marker Lamp1 (Fig. S2). We conclude that $Vps34$ activity is concentrated in a perinuclear endosomal compartment in larval fat body cells. Disruption of GFP-2xFYVE, Hrs, and Rab5 localization in $Vps34$ mutant cells indicates either that $Vps34$ activity is necessary for the recruitment or stable association of these markers or that the perinuclear endosomal compartment itself is disrupted in the mutant cells.

$Vps34$ is required for early steps of autophagosome formation

The fat body is specialized for a robust autophagic response to starvation, acting as a reserve source of nutrients for other critical tissues (Neufeld, 2004). Starvation results in an autophagy-dependent expansion and acidification of the lysosomal system, and thus the fluorescent Lysotracker dyes can be used to assay progression to late stages of autophagy in this tissue (Rusten et al., 2004; Scott et al., 2004). Starvation-induced formation of Lysotracker-positive structures was severely inhibited in clones of $Vps34$ mutant cells and in cells expressing $Vps34^{kd}$ (Fig. 2, A and B), which is consistent with a requirement for $Vps34$ in autophagy induction or progression. Overexpression of wild-type $Vps34$ was not sufficient to induce Lysotracker-positive structures under fed conditions (Fig. 2 C) but did result in a more robust response to starvation (Fig. 2 D).

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Figure 2. *Starvation-induced autophagy in the larval fat body requires Vps34*. All images depict live fat body tissue dissected from fed (C) or 4-h starved animals (all other panels). (A) Vps34Δm22 loss-of-function clones (marked by lack of GFP) fail to accumulate autolysosomes (labeled by punctate Lysotracker Red staining) after starvation. (B) Vps34Δm22-expressing cells (GFP positive) fail to accumulate punctate lysotracker Red staining under starvation conditions. (C and D) Clonal expression of wild-type Vps34 (GFP-positive cells) does not induce autophagy under fed conditions (C) but increases the response to starvation (D). (E) Vps34Δm22 loss-of-function clones (marked by lack of myrRFP) fail to accumulate autophagosomes (marked by punctate GFP-Atg8a) in response to starvation. (F and G) Starvation-induced accumulation of GFP-Atg8a punctae is observed in controls (F) but not in animals expressing Vps34Δm22 (G). (H–K) TEM images reveal abundant autophagosomes (AP) and autolysosomes (AL) in control animals (H) but not Vps34Δm22 mutants (I) nor animals expressing Vps34Δm22 (J). LD, lipid droplet. The graph in K shows autophagosome and autolysosome area ratios calculated from electron micrographs of five animals per genotype. Error bars show SD from the mean. P-values (Mann-Whitney U test): control versus Vps34Δm22, AP, 1.45e-11 and AL, 3.9e-9; and control versus Vps34Δm22, AP, 2.9e-11 and AL, 1.5e-11. Bars: (A–G) 10 μm; (H–J) 1 μm. Genotypes: (A) hsflp; FRT42D Vps34Δm22/UAS-2xeGFP FRT42D fb-GAL4, (B) hsflp; Act > CD2 > GAL4 UAS-GFP/UAS-Vps34 KD, (C and D) hsflp; Act > CD2 > GAL4 UAS-GFP/UAS-Vps34 WT, (E) hsflp; FRT42D Vps34Δm22/Cg-GAL4 UAS-GFP-Atg8a FRT42D UAS-myrRFP, (F) hsflp; Act > CD2 > GAL4 UAS-GFP-Atg8a/+ , (G) hsflp; Act > CD2 > GAL4 UAS-GFP-Atg8a/+ UAS-Vps34 KD, and (H) Vps34Δm22/CyO-GFP, (I) Vps34Δm22/Δm22, (J) Cg-GAL4 UAS-Vps34 WT /Cg-GAL4 UAS-Vps34 Δm22, and (K) as in H–J.

defect in starvation-induced autophagy (Scott et al., 2004), and we also find that mutations in the Vps15 homologue Ird1 result in a severe autophagy block (Fig. S3). To determine whether Vps34 functions in a complex containing these proteins, we first tested for physical association between epitope-tagged versions of Vps34, Vps15, and Atg6. We found that Atg6 could readily be precipitated with anti-Vps34 antibodies from larval extracts containing overexpressed myc-Atg6, Vps15-FLAG, and Vps34-TAP (Fig. 3 A). Consistent with findings in mammalian cells (Byfield et al., 2005), this association was observed under both fed and starved conditions, although starvation usually led to increased coprecipitation. Similar interactions were observed between Vps34-TAP and Vps15-FLAG (unpublished data).
To determine whether these physical associations are functionally relevant, we tested for genetic interactions between Vps34, Vps15, and Atg6. GAL4/UAS-mediated expression of Vps34-TAP resulted in a moderate statistically insignificant induction of GFP-Atg8a punctae formation under fed conditions (Fig. 3 B). Although expression of Atg6 or Vps15 also had little effect on their own, coexpression of these proteins with Vps34-TAP resulted in a significant increase in GFP-Atg8a punctae formation (Fig. 3 B). Thus, overexpression of Vps34 complexes promotes formation of GFP-Atg8a–labeled autophagosomes, but not Lysotracker-labeled autolysosomes, under fed conditions (Fig. 3 B and Fig. S3), suggesting that Vps34 activity is sufficient to drive early, but not later, steps of autophagy. Collectively, our findings suggest that D. melanogaster Vps34 is required for early events in autophagosome formation, at least in part via a conserved complex containing Vps34, Vps15, and Atg6.

Vps34 function in the endocytic pathway is distinct from its role in autophagy

Given the localization of Vps34 activity to the endosomal compartment and its role in formation of and localization to multivesicular bodies in mammalian cells (Gillooly et al., 2000; Futter et al., 2001), we next asked whether the loss of autophagy in Vps34 mutants could be attributed in part to its role in the endocytic pathway. Consistent with recent studies (Rusten et al., 2007), we found that mutations in components of ESCRT-I (Vps25), -II (Vps28), or -III (Vps32) disrupted starvation-induced Lysotracker staining in the larval fat body (Fig. 4 A; Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200712051/DC1; and not depicted). In contrast to the lack of autophagosomes in Vps34 mutants (Fig. 4 C), mutations in these ESCRT subunits led to an accumulation of GFP-Atg8a–labeled autophagosomes under fed conditions (Fig. 4 B and Fig. S4), which is consistent with a block in autophagosome-endosome fusion, as previously reported (Lee et al., 2007; Rusten et al., 2007). This difference between Vps34 and ESCRT phenotypes suggests that these mutations disrupt distinct steps of autophagy progression.

To investigate this further, we generated clones of fat body cells mutant for both Vps34 and 25 or Vps34 and 28. In these double mutant cells, GFP-Atg8a punctae still accumulated under fed conditions, albeit at somewhat reduced levels (Fig. 4 D and Fig. S4). Accumulation of autophagosomes was also evident in transmission electron micrographs of eye imaginal discs of D. melanogaster (Vaccari and Bilder, 2005), loss of Vps25 leads to accumulation of ubiquitin-positive punctae in cells of the developing eye (Fig. 4 E). Similar ubiquitin accumulation was observed in Vps34-Vps25 mutant cells, but not in Vps34 single mutants (Fig. 4, F and G), further indicating that the ESCRT pathway is not disrupted in Vps34 mutants. The ubiquitin-containing structures in Vps34-Vps25 mutant cells largely colocalized with the endosomal marker Hrs and were often adjacent to GFP-Atg8a–labeled punctae (Fig. S5). Similarly, immunofluorescence analysis of Vps25 and Vps34-Vps25 mutant eye discs revealed accumulation of ubiquitin in tubulovesicular compartments but not in autophagosomes (Fig. S5). Thus, the ubiquitinated structures in Vps25 and Vps34-Vps25 mutant cells likely represent stalled intermediates in the endocytic pathway.

Despite the relatively normal progression of ESCRT-dependent processes in Vps34 mutants, we found that they were severely defective in fluid-phase endocytosis. Vps34 mutant clones in the larval fat body failed to incorporate the endocytic tracer TR-avidin beyond the cortical region of the cell, where it often accumulated to high levels (Fig. 5 A). A similar disruption was observed in Vps34-Vps25 double mutant fat body cells (Fig. S4). Mutation of Vps34 or expression of Vps34KD also inhibited TR-avidin uptake in the highly endocytic Garland cells (Fig. 5, B–E). In addition, we observed accumulation of Notch in Vps34 mutant cells of the eye imaginal disc (Fig. 5 F), which is consistent with defective endocytic recycling (Vaccari and Bilder, 2005).

Finally, loss of Vps34 dramatically altered the distribution of GFP-Lamp1 and Spin-GFP (Fig. S2), markers which localize to...
mal rate of growth and proliferation suggests that TOR signaling is not appreciably compromised in Vps34 mutant cells. To ask whether a role for Vps34 in TOR signaling might be apparent only at higher or lower than normal levels of TOR activity, we induced clones of cells with altered expression of Rheb (a TOR activator) or Tsc1 and 2 in control or Vps34-deficient backgrounds. Cells overexpressing Rheb reached a larger size than neighboring cells, as previously reported (Saucedo et al., 2003), and coexpression of Rheb and Vps34 KD resulted in a similar cell enlargement (Fig. 6, G and H). Conversely, overexpression of Tsc1 and 2 inhibited cell growth to a similar extent in Vps34 mutant and control cells (Fig. 6, I and J). Expression of wild-type or kinase-defective Vps34 also had no effect on cell size in starved animals or animals that were allowed to recover on normal media after 48 h of starvation (unpublished data). We also tested for loss-of-function genetic interactions between Vps34 and components of the TOR pathway. Heterozygous mutation of Tor or S6k dominantly suppresses the lethality of Tsc1 homozygous mutant animals (Radimerski et al., 2002; Zhang et al., 2006), presumably by restraining the abnormally high levels of TOR signaling caused by loss of Tsc1.

Vps34 is not required for nutrient-dependent signaling to TOR

In D. melanogaster and other systems, a hallmark of TOR signaling is its stimulation of cell growth and proliferation (Arsham and Neufeld, 2006). Down-regulation of this pathway in fat body cells through Tor mutation (Scott et al., 2007), RNAi-mediated inhibition of Tor expression (Fig. 6, A and L), or overexpression of the TOR inhibitors Tsc1 and 2 (Fig. 6, B and L) results in a significant reduction in cell size. In contrast, cells mutant for Vps34 or expressing Vps34KD were similar in size to wild-type cells (Fig. 6, C, D, and L). Similarly, in the eye imaginal disc, which is comprised of mitotically active diploid cells, mutations in Tor cause a strong proliferative disadvantage (Zhang et al., 2000), whereas Vps34 mutant cells proliferate at a rate similar to that of control cells (Fig. 6, E and F). The normal rate of growth and proliferation suggests that TOR signaling is not appreciably compromised in Vps34 mutant cells.

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In contrast, Vps34 mutants had no effect on the development or viability of Tsc1 mutant animals (unpublished data).

To more directly assess the requirement for Vps34 in TOR activation, we examined the phosphorylation of S6K-Thr398, a direct substrate of the rapamycin-sensitive TOR complex I, as well as Akt-Ser505, which is phosphorylated by TOR complex II. Although the phosphorylation status of these sites is highly sensitive to the levels of Tsc1 or Rheb (Hennig et al., 2006; Scott et al., 2007), ubiquitous overexpression of UAS-driven wild-type or kinase-deficient Vps34 had no effect on the phosphorylation of these sites in whole larval extracts (Fig. 6 K). Fat body-specific expression of these transgenes also failed to alter the phosphorylation of these sites in extracts dissected fat body tissue (unpublished data). Collectively, these results indicate that in contrast to the case in cultured mammalian cells, D. melanogaster Vps34 does not play a critical role in TOR activation in vivo.

Recruitment of Vps34 activity to autophagosomal membranes is regulated by TOR/Atg1-dependent nutrient signaling

Although Vps34 is required for starvation-induced autophagy, it remains uncertain whether and how the PI(3)-kinase enzymatic activity of autophagy-specific Vps34 complexes is regulated by nutrient conditions. For example, some studies have reported an increase in Beclin 1–associated PI(3)-kinase activity in response to starvation (Byfield et al., 2005; Nobukuni et al., 2005), whereas others report a decrease (Tassa et al., 2003; Takahashi et al., 2007). Comparison of GFP-2xFYVE localization in fed and starved animals revealed that starvation induced a relative shift from a perinuclear concentration of this marker to a more widely dispersed pattern (Fig. 7, A–D and F). This did not reflect a disruption of the early endosome itself, as the TR-avidin–accessible compartment remained in the perinuclear region in starved larvae (Fig. 7, A and B). Rab5-GFP also remained associated with the perinuclear early endosome under starvation conditions (Fig. 7, C and D), and the accumulation of peripheral GFP-2xFYVE punctae was not associated with decreased perinuclear localization (Fig. 7 F).

These findings suggest that starvation causes recruitment of Vps34 activity to peripheral locations throughout the cell, presumably nascent autophagosomes. This was confirmed by the observation that the nonendosomal myc-2xFYVE punctae induced by starvation displayed a nearly complete overlap with the autophagosomal marker GFP-Atg8a (Fig. 7 E). Thus, autophagy-inducing signals result in mobilization of Vps34 activity directly to the autophagosomal membrane. Although we were unable to detect endogenous Vps34 protein in vivo, UAS-driven Vps34 did not appear to be concentrated at the early endosome nor on autophagosomes, and its localization was not affected by starvation (unpublished data). Thus, the mobilization of Vps34 activity may reflect activation of preexisting Vps34 protein at distinct cellular locations. Alternatively, the high levels of UAS-driven expression may mask more subtle changes in the distribution of endogenous protein.

TOR signaling plays a critical role in conveying nutrient status to the autophagic machinery and may directly regulate the activity of the Ser-Thr kinase Atg1 (Kamada et al., 2000; Scott et al., 2007). To determine whether this pathway is involved in regul-
including autophagy, endocytosis, endosome maturation, and both anterograde and retrograde trafficking between the Golgi and lysosome (Lindmo and Stenmark, 2006). Our findings indicate that despite these activities, the in vivo role of Vps34 in autophagy is largely limited to its function at the autophagosome. Although fluid-phase endocytosis, endocytic recycling of Notch, and trafficking of lysosomal proteins were disrupted by mutation of Vps34, our results suggest that events subsequent to autophagosome formation, including fusion between autophagosomes and endosomes or lysosomes and subsequent lysosomal degradation, are not rate limiting in the absence of Vps34.

Why does endocytic disruption lead to autophagosome fusion defects in ESCRT mutants but not in Vps34 mutants? Accumulation of endocytic tracer at the periphery of Vps34 mutant cells suggests that Vps34 functions at an early step of endocytosis,
accumulation in Vps34 single mutants indicates that ESCRT complexes are at least partially functional in the absence of Vps34. Thus, PI(3)P may not be absolutely essential for these processes, or perhaps sufficient levels of PI(3)P are generated independently of Vps34 by the class II PI(3) kinase or by PI(3,4)P or PI(3,5)P phosphatases. As ESCRT components are required for multivesicular body formation but not autophagy in yeast (Reggiori et al., 2004), it will be interesting to determine whether the requirement for ESCRT complexes in autophagy in higher eukaryotes reflects their role in multivesicular body formation or an alternate function.

The cellular compartment in which Vps34 acts to promote autophagy and the mechanisms by which it is regulated by nutrient signals have remained unresolved. In mammalian cells, and apparently this event, as well as normal endocytic flux is not essential for fusion of autophagosomes with elements of the endosomal-lysosomal compartment. Interestingly, the accumulation of autophagosomes in ESCRT/Vps34 double mutants indicates that loss of Vps34 does not completely prevent autophagosome formation. Similarly, the lack of autophagosome

Figure 7. Recruitment of PI(3)P from early endosomes to autophagosomes in response to TOR/Atg1-dependent nutrient signaling. (A and B) Internalized TR avidin colocalizes with GFP-2xFYVE under fed conditions (A) but not after 4-h starvation (B). (C and D) Starvation causes dispersion of myc-2xFYVE but not Rab5-GFP. (E) Under starvation conditions, myc-2xFYVE displays extensive colocalization with GFP-Atg8a. Yellow circles in E’ and E” show overlap between GFP-Atg8a and myc-2xFYVE label. Bar, 10 μm. (F) Quantitation of the myc-2xFYVE-labeled compartment area in a 2-μm ring surrounding the nucleus (perinuclear) and the remaining peripheral cell area (cytoplasmic). Genotypes: [A and B] Cg-GAL4/UAS-GFP-2xFYVE, [C and D] hslp; Act>CD2>Gal4 UAS-GFP/UAS-myc-2xFYVE, and [E] Cg-Gal4 UAS-2xFYVE/Aus-myc-2xFYVE.

Figure 8. Vps34 functions downstream of TOR signaling. (A and B) Starvation-induced redistribution of Vps34 activity requires TOR-Atg1 signaling. 4-h starvation causes relocalization of GFP-2xFYVE in control cells but not in Tsc2 homozygous mutant cells (A) nor in cells mutant for Atg1 (B). Mutant clones are marked by lack of dsRed. Note also the changes in GFP-2xFYVE levels in the mutant cells. [C and D] Cooverexpression of Tsc1 and 2 in GFP-marked clones induces formation of autolysosomes under fed conditions in control (C) but not Vps34 mutant (D) animals. Bar: (A and B) 10 μm; (C and D) 25 μm. Genotypes: (A) hslp; Cg-Gal4 UAS-GFP-2xFYVE/+; Tsc2^{192} FRT80B/UAS-dsRed FRT80B, (B) hslp; Cg-Gal4 UAS-GFP-2xFYVE/+; Atg1^{Δ 3D} FRT80B/UAS-dsRed FRT80B, (C) hslp; Vps34^{Δ m22}/Vps34^{Δ m22}; Act>CD2>Gal4 UAS-GFP/UAS-Tsc1 UAS-Tsc2, and (D) hslp; Vps34^{Δ m22}/Vps34^{Δ m22}/ Vps34^{Δ m22}; Act>CD2>Gal4 UAS-GFP/UAS-Tsc1 UAS-Tsc2.

The cellular compartment in which Vps34 acts to promote autophagy and the mechanisms by which it is regulated by nutrient signals have remained unresolved. In mammalian cells,
Beclin1/Atg6 has been reported to localize to the trans-Golgi network, ER, and mitochondria (Kihara et al., 2001; Pattingre et al., 2005). It was recently shown that Beclin1–Vps34 complexes can be inhibited by the antiapoptotic factor Bcl-2 in a nutrient-dependent manner (Pattingre et al., 2005). Bcl-2 mutants that are targeted to the ER, but not to mitochondria, retain their capacity to inhibit starvation-induced autophagy, suggesting that the ER is an important site of Beclin1–Vps34 regulation. However, it is unknown how these organelles contribute to the formation of autophagosomes, and recent studies suggest that rather than budding off a preexisting compartment, the autophagosomal membrane is likely to form de novo from small lipid transport vesicles or lipoprotein complexes (Juhasz and Neufeld, 2006; Kovacs et al., 2007). Our finding that myc-2xFYVE is recruited to GFP-Atg8a–positive structures under starvation conditions indicates that Vps34 activity is targeted directly to autophagosomes in a TOR/Atg1-dependent manner. Although these results do not distinguish between a role for TOR/Atg1 signaling in regulating Vps34 activity versus providing a platform on which Vps34 complexes can assemble, together with these previous studies they indicate that Vps34 is likely to promote autophagy by different mechanisms from multiple cellular locations.

How the TOR signaling pathway senses intracellular levels of nutrients, such as amino acids, has been poorly understood despite considerable work in yeast, mammalian, and other model systems. The recent identification of Vps34 as a transducer of this signal in mammalian cells thus represents a significant new insight into this issue (Byfield et al., 2005; Nobukuni et al., 2005). However, further work is necessary to determine the extent to which this mechanism is generally conserved, as starvation appears to have opposing effects on Vps34 activity in yeast versus in cultured cells as well as the experimental paradigms of these systems. For example, although complete starvation is commonly used to inactivate TOR in cell culture studies, such experiments may not accurately mimic physiologically relevant events, given the inherent capacity of intact organisms to buffer changes in nutrient levels. Additional studies in an in vivo mammalian system will be helpful to clarify these issues.

**Materials and methods**

**Fly stocks and culture**

Flies were raised at 25°C on standard carameleol/malassez agar media. The following D. melanogaster stocks were used: P(GawB)NP1626 (Kyoto stock center), Df(2R)bw5 and UAS-YFP:Rob5 (Bloomington stock center), UAS-GFP:2xFYVE, UAS-myc:2xFYVE, and UAS:Rob5:GFP (gifts of M. Gonzalez-Gaitan, Max Planck Institute, Dresden, Germany), UAS:HRP-Lamp1, UAS-GFP:Lamp1, and FRT42D Vps28R (Joseph, 2005) [gifts of M. Kramer, University of Texas Southwestern Medical Center, Dallas, TX], UAS-spin-GFP (gift of G. Davis, University of California, San Francisco, San Francisco, CA), ird1[3] and UAS:Vps15 (gifts of L. Wu, University of Maryland, College Park, MD), FRT42D Vps25A (gift of D. Bilder, University of California, Berkeley, Berkeley, CA), FRT:G3 Vps32/shrb[4] (gift of F.-B. Gao, Gladstone Institute, San Francisco, CA), UAS:Tor dsRNA line 5092R-2 (National Institute of Genetics, UAS:ts1c1 and UAS:ts2c2 (gifts of N. Tapon, London Research Institute, London, UK), Rhes[EP50.084] (gift of E. Hafen, Institute for Molecular Systems Biology, Zürich, Switzerland), Tsc2[192] FRT80B (gift of N. Ito, Massachusetts General Hospital, Charlestown, MA), Tor[ΔP] (Zhang et al., 2000), Tor[97C] (Zhang et al., 2006), and Atg1[Δ23] (Scal et al., 2005).

Null and hypomorphic alleles of Vps34 were generated as previously described (Scott et al., 2004) through imprecise excision of P(GawB)NP1626 (Hayashi et al., 2002), inserted after base 13 of the annotated PI3K_59F transcript (Flybase ID Fbj0072064). Excision events were characterized by PCR amplification of the Vps34 locus using the primers 5'-GCTGGAGGACGACAGATCCGACG-3' and 5'-CAGCAAGCTGACATCATTTAGGACGACGGC-3'.

**RT-PCR of wild-type and mutant animals**

Total RNA was isolated from 5 mg of larvae per genotype using RevertAid Total RNA isolation kit (Fermentas). cDNA was synthesized using a First Strand cDNA Synthesis kit (Fermentas) with random primers and used in PCR reactions with the Vps34-specific primers 5'-GGTGTTACCAACAGCGCAAC-3' and 5'-GTCGCCAGATCGCTTCC-3' with primers 5'-GTCGCCAGATCGCTTCC-3' and 5'-AAACGCTGAGATGGCGACACCCAC-3' specific to actin as control.

**Construction of transgenic D. melanogaster lines**

All P element constructs were injected into w- embryos according to standard procedures.

UAS-GFP-Atg8a. A NotI-XbaI fragment containing the GFP-Atg8a coding region was excised from pCasper-HS:GFP-Atg8a (Scott et al., 2004) and ligated into NotI-XbaI-digested pUAST.

UAS-Atg6. Atg6/CG5429 EST ID35669 was cloned from the vector pOT2 into pUAST using Xhol and EcoRI restriction sites.

UAS-Myc-Atg6. The Atg6 coding region was PCR amplified from EST ID35669 using the primers 5'-CACCATGATGAGGCGGAGAAA-3' and 5'-TCACGGTGACACAAAACTGTC-3', cloned into pENTR/D-TOPO, and then recombined into the D. melanogaster Gateway vector pTMW using LR clonase.

UAS-Vps34. The D. melanogaster Vps34 coding region was PCR amplified from EST GH13170 and ligated into the pUAST vector. A kinase-defective version of this construct (UAS-Vps34KD) with DBS50-5 and N810-1 substitutions was generated by site-directed mutagenesis of pUAST-Vps34 and confirmed by sequencing.

UAS-Vps34-TAP. TOPO-TA cloning was used to clone the D. melanogaster Vps34/Pi3K_59F ORF using the primers 5'-GGGGTTACCAACAGCGCAAC-3' [introducing a 5' KpnI site] and 5'-CTAGGATATCTGCTTCC-3' for N-terminal fusion and 5'-GCAGATGTATACTGCTTGGA-3' and 5'-GGTGTTACCAACAGCGCAAC-3' for C-terminal fusion. To generate N-terminal UAS-Vps34-TAP and Vps34 was cloned from TOPO-Vps34(N) into pUASTNTAP (obtained from A. Veraksa, University of Massachusetts, Boston, MA) using KpnI and XbaI restriction sites. C-terminal UAS-Vps34-TAP was generated by cloning Vps34 from TOPO-Vps34(C) into pUAST-CIAP using EcoRI and KpnI restriction sites.

**Histology and imaging**

Clonal analysis of fat body tissue and lysotracker staining were performed essentially as previously described (Scott et al., 2007), except that lysotracker Red DND-99 (Invitrogen) was used at 100 nM. TR avidin uptake was performed as previously described (Hennig et al., 2006), with pulse and chase times as indicated. For immunohistochemistry, the following antibodies were used: mouse monoclonal anti–mys (9E10; 1:1,000; gift of...
M. Stewart, North Dakota State University, Fargo, ND; mouse anti-D melanogaster Golgi (p120; 1:150; EMD); rabbit anti-Vps34 (1:300; Siddhanta et al., 1998); chicken anti-AbI (1:1000; gift of D. Bilder); chicken anti-ubiquitin (1:200; ebioscience); and mouse monoclonal anti-Notch extracellular domain (F461.3B; 1:10; Developmental Studies Hybridoma Bank). Images of live lysotracker-stained fat bodies were obtained on an epifluorescence microscope (Axioscope-2; Carl Zeiss, Inc.) with a Plan-Neofluar 40 × 0.75 NA objective, coupled to a digital camera (DXM1200; Nikon) controlled by ACT1 acquisition software (Nikon). Confocal images were acquired on a microscope (Axioplan-2; Carl Zeiss, Inc.) with a Plan-Apochromat 63× 1.4 NA objective, equipped with a CARV spinning disc confocal system and a digital camera (ORCA-ER; Hamamatsu). Axiosvision software (Carl Zeiss, Inc.) was used for acquisition, and images were further processed using Photoshop CS2 (Adobe).

GFP-Atg8 quantification
24h cultures of larvae carrying hs-GFP-Atg8a, Lsp2-GAL4, and UAS transgenes were raised to third instar, heat shocked for 1 h at 37°C, and incubated at 25°C for 3 h under feeding or starvation conditions. For starvation experiments, incubations were done in a dish with a wet kimwipe. For feeding experiments, feeding larvae were scooped, along with food, into a dish for incubations. Larval fat body was dissected in phosphate-buffered saline 3 h after completion of heat shock and mounted in SlowFade Gold antifade reagent (Invitrogen). Fat body samples were imaged with a confocal laser scanning microscope (LSM510; Carl Zeiss, Inc.) using a 40× objective. For each genotype, three fields in each of 10 animals were imaged and GFP-positive spots were counted.

Transmission EM
Tissues were dissected and fixed overnight at 4°C in 3.2% PFA, 1% glutaraldehyde, 1% sucrose, and 0.028% CaCl₂ in 0.1 N sodium cacodylate, pH 7.4, thoroughly washed in 0.1 N sodium cacodylate, pH 7.4, postfixed in 0.5% osmium tetroxide for 1 h, and embedded in Durcupan resin (Sigma-Aldrich) according to the manufacturer’s recommendations. 70-μm sections were cut, stained in Reynolds lead citrate, and viewed on a transmission electron microscope (CM-II 100; JEOL). Immuno-EM was performed as described in Juhasz et al. (2007).

TEM morphometry and Western blot
Five images per section were taken randomly at a magnification of 2,700 from five larvae per genotype (25 pictures). The area of autophagosomes, autolysosomes, and total cytoplasm was calculated using Photoshop by manually encircling relevant structures. Values are given as percent of total cytoplasm ± SD. Significance values were calculated by STATISTICA software (StatSoft) using Mann-Whitney U probes.

Immunoprecipitation and Western blot
After 1 h heat shock at 35-36°C for 3 h; 25 μl LysM buffer. Antigen–antibody complexes were released from beads with a 10-min incubation at 95°C, followed by centrifugation at 10,000 g for 5 min at 4°C. Protein was separated by SDS-PAGE on a 7.5% acrylamide gel and transferred to an Immobilon-P PVDF membrane (Millipore). Membranes were blocked in 3% milk/TBST for 1 h at room temperature and washed three times for 10 min each in TBST. Blots were incubated with primary antibody (anti-c-Myc [1:200] in 2% BSA/TBST) for 1 h at room temperature, followed by three 10-min washes in TBST. Blots were incubated in secondary anti-rabbit/HRP secondary antibody (Nikon) diluted for 1:2,000 in 3% milk/TBST for 1 h at room temperature. Blots were washed in TBST and then incubated with ECL reagent (GE Healthcare) and exposed with Hyperfilm ECL (GE Healthcare).

Online supplemental material
Fig. S1 displays the localization of early endosomal markers in wild-type and Vps34 mutant cells. Fig. S2 shows the effect of Vps34 on additional cellular markers. Fig. S3 shows the effects of Vps34 and 15α on markers of autophagy. Fig. S4 displays endocytic and autophagic phenotypes of Vps25-Vps34 mutants. Fig. S5 includes TEM and confocal analysis of Vps25-Vps34 single and double mutants. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200712051/DC1.

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