The Atg1-Tor pathway regulates yolk catabolism in *Drosophila* embryos
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Summary

Yolk provides an important source of nutrients during the early development of oviparous organisms. It is composed mainly of vitellogenin proteins packed into membrane-bound compartments called yolk platelets. Catabolism of yolk is initiated by acidification of the yolk platelet, leading to the activation of Cathepsin-like proteinases, but it is unknown how this process is triggered. Yolk catabolism initiates at cellularization in Drosophila melanogaster embryos. Using maternal shRNA technology we found that yolk catabolism depends on the Tor pathway and on the autophagy-initiating kinase Atg1. While Atg1 was required for a burst of spatially-regulated autophagy during late cellularization, autophagy was not required for initiating yolk catabolism. We propose that the conserved Tor metabolic sensing pathway regulates yolk catabolism, similar to Tor-dependent metabolic regulation on the lysosome.

Key Words: Yolk, Tor, Drosophila
Introduction

Oviparous (non-placental) embryos are closed systems that are provisioned with nutrients during oogenesis. Storage molecules supply energy and biosynthetic precursors required for early embryogenesis. These include protein in yolk platelets, as well as glycogen and lipid droplets (Gutzeit et al., 1994; Koch and Spitzer, 1982). In insects, amphibians, and many other species, yolk platelets are membrane-bounded compartments that contain a dense aggregate of specific yolk proteins, primarily vitellogenins, which form in the oocyte by endocytosis of these proteins from maternal supplies (Koch and Spitzer, 1982).

Regulation of yolk catabolism is not well understood. A leading hypothesis is that yolk degradation is triggered by decreasing pH in yolk platelets (Fagotto, 1995). Supporting this, yolk platelets become acidic during early development in *Xenopus laevis* (Fagotto and Maxfield, 1994). In *Ornithodoros moubata* (tick) eggs, Cathepsin-like proteinases within yolk platelets are activated in an acid-dependent manner (Fagotto, 1990a; Fagotto, 1990b). Similarly, in *Drosophila melanogaster*, activation of a cathepsin B protease correlates with catabolism of yolk platelets during embryogenesis (Medina, Leon, and Vallejo 1988). While these studies revealed enzymatic regulation, upstream cell signaling factors that regulate yolk catabolism during embryogenesis have not yet been identified.

The Tor pathway is a potential candidate for regulating yolk catabolism during embryogenesis because it is known to maintain nutrient level homeostasis by regulating a metabolic pathways in many systems (Kim et al., 2013b). Tor is a serine/threonine kinase regulated by amino acid abundance, the AMP/ATP sensor AMPK, and other growth signaling pathways (Bolster et al., 2002; Garami et al., 2003; Hara et al., 1998). In turn, Tor controls a number of processes including protein translation, glucose import, and autophagy, in order to
adjust metabolic activity to match available nutrient supplies (Buller et al., 2008; Hara et al., 1998; Kanazawa et al., 2004). Under nutrient-rich conditions, Tor localizes to the outer surfaces of lysosomes (Dibble et al., 2012). This recruitment is initiated by amino acids, which recruit Tor to the lysosome through Rag GTPases and regulate its kinase activity (Sancak et al., 2008).

An important downstream target of Tor is Atg1, which Tor phosphorylates and inhibits under nutrient rich conditions. Atg1, known as Ulk1 in humans, is a serine-threonine kinase whose activation triggers formation of autophagosomes (Matsuura et al., 1997). Atg1/Ulk1 is thought to initiate autophagosome formation through phosphorylation of a number of autophagy pathway proteins including the AMBRA1-PIK3C3 complex and membrane-recruiting protein Atg9 (Di Bartolomeo et al., 2010; Papinski et al., 2014). Additionally, Atg1/Ulk1 negatively regulates Tor through phosphorylation. Mutual inhibition between Tor and Atg1 creates a negative feedback loop (Dunlop et al., 2011). Ulk1/Atg1 has also been implicated in potentially non-autophagic roles. In Caenorhabditis elegans, mutation of the Atg1 homolog UNC-51 causes defects in axonal elongation and accumulation of membrane and vesicles (Ogura, 2006). Similarly, mutation of Atg1 in Drosophila causes defects in vesicular transport along neurons (Mochizuki et al., 2011).

Here, we report the requirement of Tor for activation of the Cathepsin-like protease that promotes yolk catabolism in Drosophila melanogaster embryos. Additionally, we uncovered that catabolism depends on Atg1, but is independent of autophagy. These findings shed light on how a conserved metabolic sensing pathway has been opted to regulate metabolite provision in early embryos, which are closed to nutrient import from the environment.
Results

The Tor pathway regulates yolk catabolism in early *Drosophila* embryogenesis

In *Drosophila*, three vitellogenins make up approximately 20% of the total protein in the early embryo (Bownes and Hames, 1977; Warren and Mahowald, 1979). To measure vitellogenins, we subjected total embryonic extracts from 10 embryos to SDS-PAGE and stained with Coomassie Blue. We found that vitellogenin levels, measured by densitometry of bands running at 45, 46, and 47 kiloDaltons, decreased starting at 2-3 hours post fertilization (cellular blastoderm stage) (Fig. 1A). This finding is in agreement with previous reports of the timing of vitellogenin catabolism and activation of a yolk-bound cathepsin B-like proteinase in *Drosophila* embryos (Bownes and Hames, 1977; Medina et al., 1988). Measurement of total vitellogenin in this manner is only semi-quantitative, since it is sensitive to loss of protein during sample preparation and variability in embryo size. To more quantitatively assess yolk catabolism we measured total cathepsin B-like enzymatic activity with a standard fluorogenic peptide substrate. This activity was shown to coincide with yolk degradation in *Drosophila* (Medina et al., 1988). Cathepsins are activated at low pH, and have a variable, optimum pH range for proteinase activity. We determined that cathepsin B-like enzymatic activity in *Drosophila* embryonic extract exhibits maximal activation when pre-treated at pH 3.5, and has an optimal activity range of pH 4.5-5.5 (Fig. S1). These values are similar to those previously reported by Fagotto (1990b). A previous study by Medina, Leon, and Vallejo (1988) reported that 93% of cathepsin B-like activity was in the insoluble yolk fraction during this developmental time window.
To measure the extent to which yolk catabolism has been activated, we measured cathepsin B-like activity in embryonic lysate without acid pre-treatment at pH 3.5, and normalized it to activity after pre-activation. This procedure, which was devised by Fagotto in tick embryos, measures fractional activation and also corrects for variability in embryo size and lysate preparation (Fagotto, 1990b). The fraction of cathepsin B-like that was activated was measured over the first 5 hours of development. In control embryos, 50% of total cathepsin B-like activity was already activated in 0-2.5 hour embryos, while 100% was activated in 2.5-5 hour embryos (Fig. 1B). The cathepsin B-like activity negatively correlated with a decrease in total vitellogenin (r = -0.98, p = 0.004, Fig. 1B). The fraction of cathepsin B-like already activated at early stages is higher in Drosophila embryos (~50%) than that reported in tick (~2%) (Fagotto, 1990b). This difference may reflect more rapid development in Drosophila, where yolk is utilized within 24 hours, as compared to tick eggs which consume yolk over a period of 15 days (Fagotto, 1990a).

To determine whether Tor was involved in yolk catabolism, we generated Tor deficient embryos using the maternal-Gal4/UAS-shRNA system to generate females loaded with maternal short hairpin RNAs (shRNAs) targeting Tor (Ni et al., 2011; Sopko et al., 2014), referred to as shRNA-Tor embryos. Knockdown efficiency of all shRNA lines used in this paper were quantified by RT-PCR, as reported in supplementary Table 1. Note that Tor activity in the germline is essential for growth and survival (LaFever et al., 2010; Sun et al., 2010). However, by using a maternal Gal4 driver that induces shRNA expression outside the germline stem cell compartment during stage 1 of oogenesis, we were able to bypass the early germline defects (Fig. S2C) (Yan et al., 2014). shRNA-Tor embryos were smaller than control shRNA embryos and showed significant DNA fragmentation post-cellularization, which to our
knowledge has not previously been reported as a consequence of Tor mutation or chemical inhibition (Fig. 2B, 2C, S2A). Phospho (Thr398) S6k was undetectable in both control shRNA embryos and shRNA-Tor embryos by immunoblotting, suggesting that the normal regulation of translation through S6k by Tor may not occur in embryos during this early period when they are maternally loaded with ribosomes (Fig. S2B). By introducing an EGFP-tagged Histone-2Av (His2Av-EGFP) into the shRNA-Tor line we were able to visualize the clumping and misorganization of DNA following syncytial divisions, which was not seen in shRNA-control embryos (Fig. S3A, S3B). The same phenotype was observed using two shRNAs targeting different regions of the Tor transcript, reducing the possibility that the shRNA-Tor phenotype is due to off-target effects (Fig. 2B). TUNEL staining of shRNA-Tor embryos was positive for nicked DNA after cellularization, suggesting that the fragmented DNA may be apoptosis-related (Fig. S4).

Most cathepsin B-like activity was already activated in 0-2.5 hour shRNA-Tor embryos and did not change significantly between 0-2.5 and 2.5-5 hours (Fig. 2A). Thus, Tor is necessary for regulating yolk catabolism. Tor is part of a complex, Torc1, which includes Raptor, mLST8, Deptor, and Pras40 (Loewith et al., 2002). In shRNA-raptor embryos, we observed similar, high and unchanging, cathepsin B-like activity to that of shRNA-Tor embryos (Fig. 2A). Torc1 activity also requires the GTPase, Rheb (Inoki et al., 2003; Tee et al., 2003). Similar to shRNA-Tor and shRNA-raptor embryos, cathepsin B-like activity was prematurely elevated and does not significantly increase further in Rheb depleted embryos post-cellularization (Fig. 2A). Depletion of the GTPase activating protein RagA/B, required for recruitment of Tor to the lysosome, did not affect the kinetics cathepsin B-like activity (Fig. 2A). Knockdown of Rag A/B or its heterodimer partner RagC/D was shown to only
decrease Tor activity by 50% in *Drosophila* S2 cells (Demetriades et al., 2014), and so it is possible that co-depletion of other Rags together would have a stronger effect.

To determine whether the *Tor* depleted embryos showed altered yolk platelet morphology, we examined them by thin section electron microscopy (EM). Prior to cellularization the shRNA-Tor embryos exhibited normal yolk morphology (Fig. S5A). However, during and after cellularization shRNA-Tor embryos displayed abnormal yolk morphologies, specifically decondensation of the vitellogenin mass, as compared to control embryos, and appearance of electron lucent areas within this mass (Fig. 3A-D). The diameter vitellogenin mass was also significantly larger than control embryos (Fig. 3E). This morphology, combined with early activation of cathepsin B-like protease, may indicate premature initiation of yolk catabolism.

EM also confirmed more general organizational defects seen at the light level. In control embryos we observed normal membrane ingression around the nuclei after cellularization, while in shRNA-Tor embryos we observed clumping of nuclei around the periphery of the embryos with no clear cellularization (Fig. 2C). Additionally, swaths of multivesicular, endocytic-like compartments and potentially autophagosomes were present throughout the disorganized cytoplasm (Fig. S5B). We conclude that Tor, the Torc1 complex member Raptor, and the lysosomal activating GTPase Rheb are required for normal yolk catabolism, and that Tor is also required for normal cytoplasmic morphology.

**Atg1 promotes spatially regulated autophagy**

One important role of Tor is to regulate autophagy, which serves a major role in catabolizing macromolecules to provide nutrients to cells during starvation. Autophagy has
been show to be involved in degradation of whole organelles including peroxisomes, mitochondria, and lipid droplets (Hutchins et al., 1999; Kissova et al., 2004; Singh et al., 2009). We therefore suspected an involved role of autophagy in yolk catabolism. We started by characterizing autophagy during early development in control shRNA embryos. In Drosophila, autophagy has been studied in specific tissues later in development, for instance in the developing fat body (Scott et al., 2004), but its activity during early developmental stages has not been characterized. By EM, we failed to observe autophagosomes during the syncytial divisions. Shortly after cellularization (by stage 7), abundant autophagosomes appeared, which were characterized by double bilayered compartments, 0.5-1 \( \mu \)m in diameter, often wrapped around mitochondria or lipid droplets (Fig. 4A-D). We carefully inspected micrographs for the presence of ribosomes on these membranes, to distinguish rough ER from autophagosome membranes. Multivesicular vacuoles that may represent late stage autolysosomes appeared at the same time (Fig. 4D). We also imaged the autophagosome marker, mCherry-Atg8a by immunofluorescence and observed formation of abundant puncta consistent with autophagosomes appearing shortly after the onset of cellularization (Fig. S6). Both EM and immunofluorescence revealed that autophagy is subject to tight spatial regulation in early Drosophila embryos. Autophagosomes formed primarily within a thin border, 5-7 \( \mu \)M, wide, on both sides of the ingressing cellularization front, which was also observed with the mCherry-Atg8a reporter (Fig. 4F, S6B). Very few autophagosomes were observed in the central and apical regions of the blastomeres, or within the yolk mass.

To test if the double bilayer bounded structures were indeed autophagosomes, and also evaluate the role of the autophagy pathway in their formation, we used EM to examine Atg1 deficient embryos generated by maternally expressing shRNA. We observed a drastic
reduction in formation of autophagosomes at cellularization in these embryos (Fig. 5A, 5C). This was also observed using the UAS-mCherry-GFP-Atg8a reporter combined with an shRNA against Atg1 (Fig. S7). Additionally, when we knocked down a downstream component of the autophagy pathway, Atg2, autophagosomes at cellularization were also absent (Fig. 5B, 5C, S8). Interestingly, shRNA-Atg1 embryos also exhibited disorganization of organelles. In control embryos and shRNA-Atg2 expressing embryos a layer of lipids and mitochondria forms between nuclei and yolk. In shRNA-Atg1 embryos this was not present (Fig. 5D). However, based on staining with the cellularization marker anillin, shRNA-Atg1 embryos appear to cellularize normally (Fig. S9). We conclude that autophagy is activated at the cellular blastoderm stage, that it triggers formation of abundant autophagosomes in a selective region spanning the cellularization front, and Atg1 also controls organelle patterning in an autophagy-independent manner.

**Atg1, but not autophagy, is required for normal yolk catabolism**

Given that both yolk catabolism and autophagy are initiated during the cellular blastoderm stage, and both are Tor-regulated, we next investigated whether autophagy is required for yolk catabolism. We measured cathepsin B-like activity in embryos expressing shRNA targeting Atg1 and Atg2, which by EM were both deficient in autophagosomes (Fig. 5C). Surprisingly, Atg1, but not Atg2, was necessary for timely activation of cathepsin B-like protease activity (Fig. 6A). Additional shRNAs against other autophagy proteins, Atg4a, Atg5, and Atg10, also had no affect on cathepsin B-like enzyme activity compared to control embryos. We also tested Fip200$^{35S/3F5}$ (Atg17) mutant embryos. Fip200 forms a complex with Atg1 and is required for autophagy (Hara et al., 2008). Fip200 homozygous mutants survive
until the P15 pupal stage, however die before eclosion (Kim et al., 2013a). Fip200\(^{35S/3F5}\) embryos showed similar cathepsin B-like activity as \(shRNA-Atg1\) embryos demonstrating that Atg1 complex, not just the Atg1 kinase, is required for yolk catabolism. Interestingly, the cathepsin B-like basal activity of \(shRNA-Atg1\) and \(Fip200^{35S/3F5}\) embryos was higher than control \(shRNA\) embryos, but not as high as the basal activity of \(shRNA-Tor\) embryos. When both pre-and post-cellularization activity were plotted on a 2D graph, embryos deficient for Atg1 complex components (Atg1 and Fip200) show different activation levels compared to both the control embryos and Tor pathway knockdowns (Fig. 6B). Coomassie staining of \(shRNA-Atg1\) compared to control showed no change in yolk vitellogenins between 0-2.5 HPF and 2.5-5 HPF, similar to \(shRNA-Tor\) embryos (Fig. S10). However, based on this staining we can only measure relative levels over time, we cannot determine whether total yolk levels differ. Based on these findings, we determined that the Atg1 complex is necessary for yolk catabolism, but autophagy is not required. Additionally, \(Atg1\) knockdown affects yolk catabolism in a distinctly different manner from knockdown of components in the Tor pathway.

**\(Atg1\) knockdown rescues Tor-\(shRNA\) expressing embryos**

\(Atg1\) and Tor have previously been shown to mutually inhibit each other (Dunlop et al., 2011), so we hypothesized that knocking down Tor leads to over-activation of Atg1, and vice versa. To test this, we constructed a line simultaneously targeting \(Atg1\) and Tor (\(shRNA-Tor\); \(shRNA-Atg1\)). Strikingly, depletion of \(Atg1\) rescued both the morphology and hatch rate defects of \(shRNA-Tor\) embryos (Fig. 5C, Fig. S11A). The \(shRNA-Atg1\); \(shRNA-Tor\) double knockdown embryos were compared to single \(shRNA-Tor\) and \(shRNA-Atg1\) knockdowns.
combined with control shRNAs to control for any effects of combining multiple shRNAs (Table S1). Additionally, when we overexpressed Atg1 we observed a similar phenotype to shRNA-Tor embryos, including positive TUNEL staining (Fig. 6D, Fig. S11B). To determine whether the rescue was due to a decrease in autophagy or an alternate activity of Atg1, we looked at the effect of knocking down Atg2. Expression of shRNA-Atg2 failed to rescue shRNA-Tor (Fig. 6C). Altogether, the double shRNA knockdowns demonstrates that Tor and Atg1 interact in a manner separate from autophagy, and that Atg1 has a separate function other than autophagy, which could be regulation of Tor.

Discussion

Our study revealed new aspects of Tor/Atg1 biology, as well as providing progress on how yolk catabolism is regulated. The dramatic Tor knockdown phenotype, with profound disorganization of the embryo, and its nearly complete rescue by Atg1 knockdown, are novel results from our study. Several controls, including measurement of mRNA levels, use of multiple shRNAs, and failure to rescue Tor depletion by Atg2 knockdown, attest to the specificity of these effects. These findings complement and extend previous studies on Tor and Atg1 function in later Drosophila development that relied on somatic mutations, where perdurance of maternally loaded protein complicates analysis (Lee et al., 2007; Scott et al., 2004). Previous studies examining rescue of Tor mutants by knocking down or mutating Atg1 reported mixed results: Scott et al. (2004) observed that a zygotic Tor−/−; Atg1−/− mutant was less viable the zygotic Tor−/− mutant. However, Lee et al. (2007) found that homozygous Tor−/− larvae, that usually die at second/early third instar larval stage, in a heterozygous Atg1 mutant background developed through mid-late third instar larval stage. Given that Atg1 and Tor are
in a negative feedback loop, one possibility is that simultaneously decreasing the activity of Tor and Atg1 prevents over-activation of either kinase. The remaining amount of Tor and Atg1 protein still present may then be able to elicit a normal phenotype. Both knockdown of Tor and overexpression of Atg1 resulted in positive TUNEL staining, suggesting apoptotic-related cell death (Fig. S4, S11). Previous work overexpressing Atg1 in the wing imaginal disks and fat body also observed cell death and positive TUNEL staining (Scott et al., 2007). Interestingly, the work by Scott et al. 2007 was able to reduce Atg1-induced cell death through inhibition of autophagy. Our study found that shRNA-Tor embryos can be rescued through inhibition of Atg1, be we were not able to rescue shRNA-Tor embryos by inhibiting another downstream autophagy gene, Atg2, suggesting that Atg1 and Tor may have additional functions in the early embryo (Fig.6). Particularly, Atg1 has multiple roles that include initiating autophagy and promoting yolk catabolism. Both turn on dramatically at the end of cellularization, but depend on different downstream components since autophagy, but not yolk catabolism, are blocked by knockdown of Atg2.

An interesting finding of our work is the timing and spatial regulation of autophagy during cellularization. In exploring Atg1’s role in promoting autophagy in the early embryo, we documented formation of abundant autophagosomes spanning the basal region of blastomeres at the end of cellularization, coinciding with the initiation of zygotic transcription. In mouse embryos, autophagosomes also form around the period of zygotic transcription, after the first cleavage division (Tsukamoto et al., 2008). Given this timing, autophagosomes may play a role in degradation of maternal proteins during the mid-blastula transition, and/or in providing nutrients to the developing embryo by catabolism of cytosol, organelles, lipid droplets and glycogen granules. Consistent with a metabolic role, we frequently observed
mitochondria and lipid droplets inside autophagosomes by EM (Fig. 4C-D). Autophagosomes were only formed within a narrow 6 μm wide zone spanning the basal region of blastomeres (Fig. 4F). The significance of the high degree of spatial regulation is unclear. It might serve to specifically degrade molecules that would otherwise impede gastrulation, or to protect apically localized factors such as mRNAs. More detailed analysis of maternal proteins could potentially address these questions. We additionally observe the misorganization of organelles in shRNA-Atg1 embryos that is not seen in control shRNA or other autophagy-deficient embryos. Previous studies have shown that Atg1 phosphorylates a myosin light chain kinase, which could potentially affect organelle distribution through disrupting the actin-associated myosin II (Sopko et al., 2014; Tang et al., 2011).

A major finding of our work concerns regulation of yolk catabolism by the Tor pathway and Atg1. First a caveat; for molecular analysis we used activity levels of Cathepsin-B like enzyme activity as a surrogate for measuring catabolism itself. In repeated experiments we found direct measurement of vitellogenin was inaccurate, mainly because it was hard to normalize, and thus did not take into account variation in embryo size. For Cathepsin B-like activity we normalized measured activity to maximal activity following an acid treatment. This approach was developed by Fagotto (1990a, 1990b), and we found it gave consistent measurements. This is the first molecular clue as to how yolk catabolism is triggered, and also an important new function for Tor and Atg1. Studies from a variety of organisms suggest that yolk platelets already include cathepsin-like proteases that are capable of catabolizing vitellogenins, but these are stored as pro-enzymes, and must be activated by acidification of the yolk platelet from a resting pH of ~5.5 in oocytes to ~4.5 to trigger catabolism (Fagotto and Maxfield, 1994; Medina et al., 1988). This process is controlled throughout development.
such that each lineage in the embryo can trigger yolk catabolism at the appropriate time, and thus maintain the appropriate amount of nutrients prior to hatching and feeding (Jorgensen et al., 2009). How the embryo regulates the timing of yolk catabolism had not been elucidated, and it has been unclear if degradation is triggered as a developmental event, or in response to nutrient demand in different lineages. Given that the Tor pathway measures metabolism and regulates catabolism in response to nutrient demand, we speculate that Tor inactivation triggers yolk catabolism in response to nutrient demand, though we cannot rule out developmental regulation of the Tor-Atg1 circuit. Atg1 integrates input from the energy sensor AMPK to activate downstream autophagy (Kim et al., 2011). Given autophagy and yolk catabolism occur at similar times in the *Drosophila* embryo, Atg1 may potentially act as a hub to coordinate autophagy and yolk catabolism simultaneously. Previous work has also demonstrated that Atg1 can inhibit activity of the MAP kinase ERK in neurons, and that this activity is required for protein composition at the synapses (Wairkar et al. 2009). Therefore, Atg1 could also affect yolk catabolism indirectly through additional downstream signaling pathways.

Our finding of an essential role of Tor in promoting yolk catabolism opens the door to molecular analysis. Given that Rheb is also required for yolk catabolism, a lack of amino acids may activate Tor on the surface of yolk platelets, as it does on the surface of lysosomes in somatic cells. Proteomics of yolk platelets from *Xenopus Laevis* identified Rheb on the yolk membrane, supporting the hypothesis that Tor and Rheb may be localized to the yolk platelet similar to that of a lysosome (Jorgensen et al., 2009). Based on Fagotto’s “sleepy lysosome” hypothesis, we propose that the Atg1-Tor signaling axis detects amino acid demand (and or is subject to developmental regulation) and then acts to decrease the pH of yolk platelets, which
in turns activates a cathepsin protease (Fig. 7) (Fagotto, 1995). An important link connecting the Atg1-Tor signaling and regulation of lysosomal pH may be the regulation of H\(^+\)-ATPase on the lysosome membrane. It been demonstrated H\(^+\)-ATPase interacts with both the Ragulator and the RAGs, and the interactions are strengthened by amino acid starvation and weakened by amino acid stimulation (Bar-Peled et al. 2012; Zoncu et al. 2011). There is also evidence for Tor affecting lysosome pH, namely that suppression of Tor activity through starvation or Tor-specific catalytic inhibitors leads to a drop in pH, and consequently lysosome activation (Zhou et al., 2013). However, regulation of H\(^+\)-ATPases is generally a poorly understood phenomena. In yeast, the vacuolar H\(^+\)-ATPase can reversibly disassemble and reassemble the V0 and V1 complex in order to regulate activity. For example, intact, active V-ATPase complexes are disassembled into free V1 and V0 complexes in response to glucose deprivation, the can reassemble upon readdition of glucose (Kane 1995). However, a clear mechanism linking H\(^+\)-ATPase regulation and metabolic signaling has not identified in multi-cellular organisms. Yolk may provide a particularly good system for understanding the link between the Tor pathway and pH regulation of organelles. Yolk platelets have a much wider pH range (from pH 4.5 – 6.5) and are much larger (1.5-3 μM) compared to lysosomes where the pH is thought to be more tightly regulated between pH of 4.5 and 4.8 and range between 0.1-1 μM (Fagotto and Maxfield 1994; Ohkuma and Poole 1978). Developing an in vitro system where one could chemically perturb Tor or Atg1 activity and then quantitatively measure the pH of yolk platelets could be one way to answer this question. Exactly how Tor and Atg1 turn on yolk acidification is an important future question, which may have implications for control of lysosomal activity more generally.
Methods

Fly stocks

The *UAS-shRNA* and Gal4 fly stocks used in this study were obtained from the TRiP and Bloomington’s Drosophila Stock Center (BDSC). For maternal *UAS-shRNA* expression, we used either the maternal triple-driver MTD-Gal4 (BDSC 31777), a dual *MAT-Gal4* (constructed from BDSC 7062 and BDSC 7063), and a single *MAT-Gal4* (BDSC 7063). mRNA knockdown efficiencies and primers are listed in Supplementary Table 1. For a control *shRNA* we used a hairpin against *white* mRNA (HMS00017), except for with the double knockdowns in which we used hairpins against EGFP because they were available on both chromosomes (BDSC 41550, BDSC 41551). To visualize histones we P{w[+mC]=His2Av-EGFP.C}2/SM6a into *shRNA-Tor* and control *shRNA* expressing lines (BDSC 24163). The *FIP200*3F5/3F5 line was graciously provided by Hong-Wen Tang. The UAS-*HA-Atg1* overexpression construct was assembled by amplifying *Atg1* from BDSC 51654 and inserting it into pVALIUM20 with an HA sequence on the N-terminus (TRiP, Harvard Medical School). Transgenic lines were generated by Genetic Services (Cambridge, MA).

Embryo collections and hatching rates

Eggs were collected from *shRNA/Gal4* females crossed to *shRNA* males. Flies were fed molasses and yeast paste. All embryos were collected at 27 °C and dechorionated by soaking in 50% bleach for 3-5 minutes followed with extensive washing in distilled water. Embryos were washed in PBS with 0.1% Triton-X and staged on a dissecting microscope. Embryos were snap frozen in liquid nitrogen and stored at -80°C until use. For hatching rates 20 females and 10 males were placed in vials at 27°C for 18 hours. Embryos were collected from
the vials and plated on molasses agar plates. The plates were incubated at 27°C for 24 hours and hatched embryos were counted. All hatching rates were done in biological duplicates.

**Immunostaining and Western blots**

For immunostaining, collected embryos were fixed in 1:1 dilution of methanol and heptane. Embryos were shaken vigorously to remove the perivitelline layer and then transferred to 100% methanol. Methanol was exchanged 3 x followed by PBST (PBS + 0.1% Triton-X). Hoechst (1 mM) was added and then the embryos were gently rocked in the dark for 10 minutes. Embryos were washed with TBST then, transferred to a slide and mounted with ProLong Gold Mounting Reagent (Invitrogen). Embryos were imaged using a Nikon A1R point scanning confocal. For immunoblotting, embryos were lysed directly in NuPage SDS loading buffer with 50 mM DTT and lysed using a plastic pestle. Antibodies were used at the following concentrations: Phospho (Thr398) p70 s6K (Cell signaling) 1:500.

**TUNEL Staining**

TUNEL staining was carried out using TMR red labeling in situ Cell Death Detection Kit (Roche). Embryos were fixed in a 1:1 mixture of 4% formaldehyde and heptane, washed 2x with heptane, then transferred to a 1:1 mixture of methanol:heptane to remove the perivitelline membrane. Embryos were transferred to 100% methanol, then to 100% ethanol, then rehydrated in 3:1, 2:1, 1:1, 1:2 mixtures of ethanol:PBS, then finally transferred to PBS with 0.1% Triton-x. Staining was carried out according the manufacturer’s protocol (Roche).

**Thin Section Electron Microscopy**

Embryos were dechorinated for 3 minutes in 50% bleach, fixed with gluteraldehyde, thin sectioned (~60-70 nm sections) and stained with lead citrate using a previously described
method (Rickoll, 1976). Sections were imaged on a Tecnai G² Spirit BioTWIN. Autophagosomes were quantified by dividing the total sum of the area of double membrane structures that were fully enclosed per total area (~25000 μM²) of the section. Sections from three embryos were counted for each shRNA line and developmental time point. For the control shRNA stage 7 embryos, ~50-75 autophagosomes were measured for each replicate embryo.

**Quantitative real-time PCR (qPCR)**

Total RNA was isolated from ~400 embryos collected from 0-4 hour eggs from Mat-Gal4/shRNA females using Ambion Purelink RNA Mini Kit following manufacturer’s protocol. cDNA was generated from 500 ng of purified RNA with the iScript cDNA Synthesis Kit (Bio-Rad). Samples were measured in triplicate using iQ SYBR Green Supermix (Bio-Rad) on a Bio-Rad CFX96 Real Time PCR machine. Transcripts were normalized using ribosomal protein L32, alpha-tubulin, and Gapdh1 as reference genes, then compared to shRNA-white control embryos. Primers were designed using FlyPrimerBank (Hu et al., 2013) (Table S1).

**Yolk Measurements**

For Coomassie Blue staining, embryos were collected in 1-hour increments from 0-5 hours. Embryos were staged using a dissecting microscope and 10 embryos were frozen in triplicate for each line and time point. Embryos were flash frozen and stored at -80 °C until use. Samples were lysed in NuPage LDS loading buffer with 50 mM DTT and a plastic pestle. Samples were loaded onto Bis-Tris gels (Bio-Rad) and stained with Coomassie Blue. The cathepsin B-like assay was followed as described by Fagotto (1990). Briefly, samples (50-150 embryos) were lysed in 100 μL 10 mM sodium acetate, pH 5.5 and split into two tubes. The
non-acid treated sample was diluted 10-fold in 400 mM sodium acetate, pH 5.5, 4 mM EDTA and 4 mM DTT. Samples were incubated for 1 minute at 30°C. Acid treated samples were diluted 10-fold in 100 mM sodium formate, pH 3.5, 2 mM EDTA, and 1 mM DTT then incubated for 5 minutes at 30°C. 20-50 μL of the samples was then added to the substrate assay buffer (100 mM sodium acetate buffer, pH 5.5, 0.05% Brij, 2 mM DTT, 1 mM EDTA, 5 uM Z-Phe-Arg-AMC (Bachem)) and incubated for 5 minutes at 30°C. The reaction was stopped with 100 mM monochloracetate and 100 mM sodium acetate, pH 4.5. The samples were measured on a Perkin-Elmer Plate Reader using 360 nm excitation and 460 nm emission filters.

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Competing interests

The authors declare no competing or financial interests.
Author contributions

H.K., T.M. designed the experiments. H.K. performed immunofluorescence and Cathepsin experiments. N.B. and R.S. provided genetic tools and feedback. R.S. measured and analyzed shRNA efficiencies. P.C. performed electron microscopy. H.K. wrote the manuscript.
Yolk catabolism is correlated with Cathepsin B-like activation

A. Vitellogenin levels of control shRNA embryos normalized by 1 HPF yolk intensity based on Coomassie Blue staining. B. Percent active cathepsin B-like in control shRNA embryos over the first 5 hours of development. Yolk concentration and cathepsin B-like enzyme activity were negatively correlated (Pierson's correlation, $r = -0.98$, $p = 0.004$). HPF: Hours post fertilization.
Fig. 2

**Tor is necessary for yolk catabolism.** A. Percent active cathepsin B-like in shRNA-expressing embryos pre-cellularization (0-2.5 hours) and post-cellularization (2.5-5 hours). B. Phenotype of stage 7 shRNA-Tor embryos using two different hairpins. C. Staining of control shRNA and shRNA-Tor embryos with the cellularization marker anillin.
Fig. 3

Depletion of *Tor* leads to aberrant cellularization and abnormal yolk morphologies. A. EM of control *shRNA* embryos post-cellularization. Left scale bar 10 μM. Right: magnification of inset, scale bar 2 μM. B. Yolk platelets in control *shRNA* embryos. Upper
scale bar 2 μM. Lower scale bar 500 nm. C. EM of shRNA-Tor embryos post-cellularization. Left scale bar 10 μM. Right: magnification of inset, scale bar 1 μM. D. Yolk platelets in shRNA-Tor embryos. Upper scale bar 2 μM. Lower scale bar 500 nm. E. Diameter of yolk in control versus shRNA-Tor embryos (μM). The longest diameter of the darkly stained EM portion of the yolk was measured since it represents the most energy dense portion of the yolk platelets. *** = p < 0.01. N= Nuclei, L= Lipid droplet, M= Mitochondria.
Fig. 4

**Autophagy is initiated after cellularization and is spatially regulated.** A. EM images of control shRNA embryos at stage 5 (pre-cellularization) and stage 7 (post-cellularization). Scale bar: 10 μM. B. Magnification of stage 5 embryos. Scale bar: 500 nM. C. Magnification of
stage 7 embryos with an autophagosome containing a lipid droplet (asetrix). Scale bar: 500 nM. D. From left to right, examples of a phagopore, an autophagosome containing no organelles, an autophagosome containing mitochondria, and a multivesicular, endocytic-like structure. E. Percent autophagosomes per \( \mu \)M\(^2\) in pre- and post-cellularization embryos. 3 biological replicates (embryos were counted per stage). E. Cumulative distribution histogram of autophagosomes location within stage 7 embryo. Autophagosomes were classified as located either in the yolk area (negative) or cell area (positive) based on their proximity to the cellularization border.
Fig. 5

Atg1 is necessary for autophagy after cellularization. A. EM of stage 7 shRNA-Atg1 embryos and B. stage 7 shRNA-Atg2 embryos. Scale bar: 2 μM C. Number of autophagosomes per μM² in control shRNA versus shRNA-Atg1 and shRNA-Atg2 stage 7 embryos. D. Stage 5 embryos in control shRNA, shRNA-Atg1, and shRNA-Atg2 embryos. Double headed arrow: section of lipids and mitochondria in between nuclei and yolk in control shRNA and shRNA-Atg2 embryos. Arrow: displaced organelles in shRNA-Atg1 embryos. Scale bars: 10 μM.
**Fig. 6**

**Atg1, but not autophagy, is required for yolk catabolism and is regulated in a Tor-dependent feedback loop.** A. Percent active cathepsin B-like in embryos pre-cellularization (0-2.5 hours) and post-cellularization (2.5-5 hours). FIP200^{3F5/3F5} are mutant embryos, not shRNA. G. Pre- versus post- cathepsin B-like activation in shRNA expressing embryos. Control, Atg1 complex, and Tor pathway components segregate into three different clusters. C. Hatching rates of double shRNA knockdowns. D. Overexpression of *Atg1* compared to control shRNA embryos.
Fig. 7

**Model of yolk catabolism.** Atg1 is necessary for both autophagy, via downstream Atg proteins, and yolk catabolism. Its role in yolk catabolism could be as via direct regulation of Tor. Tor, its Torc1 complex member Raptor, and its activating GTPase Rheb are also required for yolk catabolism, suggesting they may be localized on the yolk platelet membrane similar to a lysosome. An important missing link may be regulation of the H⁺-ATPase by the Tor-Atg1 circuit.
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