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The Role of Autophagy in *Drosophila* Metamorphosis

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Abstract

Macroautophagy (autophagy) is a conserved catabolic process that targets cytoplasmic components to lysosomes for degradation. Autophagy is required for cellular homeostasis and cell survival in response to starvation and stress, and paradoxically, it also plays a role in programmed cell death during development. The mechanisms that regulate the relationship between autophagy, cell survival, and cell death are poorly understood. Here we review research in *Drosophila* that has provided insights into the regulation of autophagy by steroid hormones and nutrient restriction and discuss how autophagy influences cell growth, nutrient utilization, cell survival, and cell death.

1. INTRODUCTION

All animals transition through several different stages during their development. The first stage is embryonic development, followed by a juvenile growth phase, then sexual maturation, and finally reproductive adult-hood. Some animals, such as mammals, exhibit few changes in their body plan during development except for growth; human babies look like miniature adults. Other animals go through drastic changes, such as the frog, starting its juvenile phase as a tadpole and developing into a frog. Progression through the developmental stages requires the coordination of cell and tissue growth, cell survival, and cell death. In the example of the frog, cell growth allows the limbs to develop, and cell death causes the regression of the tadpole's tail. The balance of cell growth, survival, and death is critical to maintaining homeostasis of the organism.

Autophagy is a catabolic process that functions at the crossroads of the different cell fates. Autophagy is predominately associated with cell survival in response to cellular stress; however, mounting evidence suggests that it also plays a role in programmed cell death. Additionally, autophagy is regulated by the same pathways that control cell growth. Autophagy and the pathways that regulate it have been studied extensively in the fruit fly *Drosophila melanogaster*. These studies have provided insights into the relationship between cell growth, cell death, and autophagy, but the questions of how and why these signals are integrated remain poorly understood.

2. AUTOPHAGY

Autophagy is an important catabolic process in all eukaryotic cells. There are three known types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (Klionsky, 2005). Macroautophagy (hereafter referred to as autophagy) is the best characterized of the three types, and it involves the sequestration of cytoplasmic components and long-lived proteins into lysosomes for degradation. During autophagy, an isolation

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membrane sequesters cytoplasmic material, and it elongates to form a double-membrane vesicle, the autophagosome (Fig. 4.1). The autophagosome traffics to the lysosomal compartment where its outer membrane fuses with lysosomes and releases the inner cargo for degradation. Lysosomal permeases then recycle the degradation products back to the cytoplasm (Mizushima & Komatsu, 2011). Autophagy is an important process for maintaining cell homeostasis, responding to stress, and surviving nutrient starvation.

2.1. Regulatory pathways

Several metabolic regulatory factors affect autophagy induction, including nutrient availability, insulin signaling, and ATP levels (Meijer & Codogno, 2004). The mechanistic target of rapamycin (TOR) plays a central role in autophagy by integrating the class I phosphatidylinositol-3-kinase (PI3K) and amino acid signaling pathways (Wullschleger, Loewith, & Hall, 2006). When nutrients are available, class I PI3K activates TOR, which represses autophagy by phosphorylating Atg13. This hyperphosphorylation reduces the affinity of Atg13 for Atg1, decreasing the kinase activity of Atg1 and inhibiting autophagy (Kamada et al., 2000; Noda & Ohsumi, 1998). During nutrient starvation, TOR activity is reduced, relieving its repression of Atg1, and autophagy is induced. Increased autophagy contributes to cell survival by producing amino acids and fatty acids that are used by the tricarboxylic acid cycle to generate ATP (Lum et al., 2005).

The origin of the autophagic membrane is not completely understood and remains a subject of debate (Juhasz & Neufeld, 2006). In yeast, autophagy proteins gather at the pre-autophagosomal structure (PAS) near the vacuole (Mizushima, 2007). In animal cells, a PAS-like structure has never been observed. Some studies suggest that in mammalian cells, the autophagosomal membrane originates from the endoplasmic reticulum (Axe et al., 2008; Dunn, 1990). In addition, more recent research suggests that autophagosome formation involves membrane derived from the mitochondria or the plasma membrane (Hailey et al., 2010; Ravikumar, Moreau, Jahreiss, Puri, & Rubinsztein, 2010).

Formation of the autophagosomal membrane requires phosphorylation of phosphatidylinositol. In yeast, this is accomplished by a class III PI3K complex consisting of Vps30/Atg6 /Beclin1, Vps34/ class III PI3K, Atg14, and Vps15 (Kametaka, Okano, Ohsumi, & Ohsumi, 1998; Kihara, Noda, Ishihara, &Ohsumi, 2001; Suzuki et al., 2001). Atg6 also forms a complex required for the vacuolar protein sorting (VPS) pathway in yeast, which consists of Atg6, Vps35, Vps15, and Vps38(Kihara et al., 2001).The Beclin 1–Vps34 complex is similar in mammalian cells; however, it contains additional regulators, including UVRAG, Bif1, Ambra1, and Barkor (Fimia et al., 2007; Liang et al., 2006; Sun et al., 2008; Takahashi et al., 2007). As in yeast, it has been suggested that Beclin1 forms at least two distinct complexes in animal cells that play different roles in membrane trafficking (Itakura, Kishi, Inoue, & Mizushima, 2008).

2.2. Autophagosome formation

Genetic studies in yeast have identified several *Atg* genes that are required for autophagy (Harding, Hefner-Gravink, Thumm, & Klionsky, 1996; Harding, Morano, Scott, & Klionsky, 1995; Klionsky et al., 2003; Thumm et al., 1994; Tsukada&Ohsumi, 1993). Many of these genes are involved in two conserved ubiquitin-like conjugation systems that are required for autophagosome formation, Atg12 and Atg8 (LC3 in mammals) (Klionsky & Emr, 2000; Ohsumi, 2001). Atg12 and Atg8 are both activated by the E1-like enzyme Atg7. Atg12 is then transferred to the E2-like enzyme Atg10. Finally, Atg12 is conjugated to Atg5 and forms a complex with Atg16 on the isolation membrane (Kuma, Mizushima, Ishihara,&Ohsumi, 2002;Mizushima, Noda,&Ohsumi, 1999;Mizushima et al., 1998; Shintani et al., 1999; Tanida et al., 1999). Atg8 is transferred to the E2-like enzyme Atg3

and is then conjugated to the phospholipid anchor phosphatidylethanolamine (PE) (Ichimura et al., 2000). This final conjugation results in the anchoring of Atg8-PE to the isolation membrane and is thought to regulate the elongation of the isolation membrane (Nakatogawa, Ichimura, &Ohsumi, 2007). In addition to Atg7 and Atg3, Atg8 modification requires Atg4, a cysteine protease that processes Atg8 before conjugation and cleaves Atg8 from PE once the autophagosome has fused with the lysosome (Ichimura et al., 2000). Since Atg8 remains on the membrane throughout autophagosome maturation, it is a useful marker of autophagosomes (Klionsky et al., 2008).

3. *DROSOPHILA* AS A MODEL FOR STUDYING THE INTERFACE BETWEEN STEROID SIGNALING, NUTRITION, AND GROWTH DURING DEVELOPMENT

Drosophila development provides a useful system for studying the coordination of cell growth, division, and death that is necessary for the animal to reach its proper size. Fly development is regulated by the steroid 20-hydroxyecdysone (ecdysone), and insulin and insulin-like growth factor signaling. These pathways are also known to regulate autophagy in different contexts; however, the coordination of steroid, insulin signaling, and autophagy is poorly understood. Recent studies have investigated the relationship between ecdysone and growth factor signaling in flies (Colombani et al., 2005; Layalle, Arquier, & Léopold, 2008), and understanding how these two pathways coordinate with each other may provide insight into how autophagy fits into this dynamic to facilitate animal homeostasis.

3.1. Steroid signaling

During development, *Drosophila* transitions through many different stages, and these transitions are signaled by pulses of the steroid hormone, ecdysone (Riddiford, Cherbas, & Truman, 2000; Thummel, 2001). *Drosophila* begins life as an embryo, and approximately 1 day after egg lay, they hatch as first instar larvae. The larvae feed and grow for approximately 3.5 days, and they molt twice during this period to become second instar larvae 24 h after hatching and third instar larvae 48 h after hatching. After the larval period, the animal stops feeding and a high titer pulse of ecdysone triggers puparium formation. This ecdysone pulse also induces the programmed cell death of the larval midgut (Lee, Cooksey, & Baehrecke, 2002). Prepupal development lasts for 12 h, and another peak in ecdysone titer triggers the prepupal–pupal transition and initiates programmed cell death of the larval salivary glands (Lee et al., 2003). Pupal development lasts for 3.5 days, after which the adult animal ecloses. A remarkable transformation occurs during this final developmental period; the tissues necessary to the feeding larva degrade through histolysis and are replaced by growing tissues that will be necessary to the walking, flying, and reproducing adult.

Ecdysone signaling has been studied extensively in the larval salivary glands of *Drosophila*. The pulses of ecdysone regulate stage- and tissue-specific developmental pathways through a transcriptional hierarchy (Thummel, 1995) (Fig. 4.2). Ecdysone signals by binding its receptor which is a heterodimer of two nuclear receptors, ecdysone receptor (EcR) and ultraspiracle (USP) (Koelle et al., 1991; Thomas, Stunnenberg, & Stewart, 1993; Yao, Segraves, Oro, McKeown, & Evans, 1992). The EcR complex activates transcription of the early genes; these include *Broad Complex* (*BR-C*), *E74A, E75*, and *E93* (Baehrecke & Thummel, 1995; Burtis, Thummel, Jones, Karim, & Hogness, 1990; DiBello, Withers, Bayer, Fristrom, & Guild, 1991; Segraves & Hogness, 1990). The early genes then activate transcription of the late genes, which are thought to function more directly in the regulation of developmental processes. In the salivary glands, the βFTZ-F1 orphan nuclear receptor is expressed during the mid-prepupal dip in ecdysone titer (Lavorgna, Karim, Thummel, &Wu, 1993). During the ecdysone peak that triggers salivary gland degradation, the EcR complex

and βFTZ-F1 function together to reinduce transcription of *BR-C, E74A*, and *E75* and to activate transcription of the stage-specific early gene, *E93* (Baehrecke & Thummel, 1995;Broadus, McCabe, Endrizzi, Thummel, & Woodard, 1999; Woodard, Baehrecke, & Thummel, 1994). β*FTZ-F1, BR-C, E74A*, and *E93* are all necessary for the proper degradation of larval salivary glands (Broadus et al., 1999; Jiang, Lamblin, Steller, & Thummel, 2000; Lee et al., 2000; Restifo and White, 1991). *E93* may have a more prominent role in autophagic cell death than the other early genes as it also appears to be required for autophagosome formation in the dying larval midgut (Lee, Cooksey, & Baehrecke, 2002).

3.2. Growth and nutrient utilization

Growth regulation at the cellular, tissue, and organismal level is critical for proper size development in all multicellular organisms, and it is affected by several environmental factors including nutrient availability (Mirth & Riddiford, 2007). In *Drosophila*, the feeding larva grows an astounding amount, increasing its size by ~200-fold during the 3.5-day period (Church & Robertson, 1966). Without this accumulation of body mass, the fly may have reduced reproductive success as an adult or it may not even be able to survive metamorphosis from the larva to adult.

For the adult fly to reach its proper size, the larva must pass three weight checkpoints. The first checkpoint occurs near the second instar to third instar molt and is called the threshold size for metamorphosis (Zhou, Zhou, Truman, & Riddiford, 2004). This size assessment determines whether the next molt will be a larval or metamorphic molt (Nijhout, 1975). The second checkpoint is the minimal viable weight which is the minimum body mass that is necessary to complete larval and pupal development in the absence of nutrients (Bakker, 1959). The final checkpoint, critical weight, occurs during the last larval stage (Nijhout, 2003; Nijhout &Williams, 1974). Reaching critical weight ensures that the animal will pupate within a certain amount of time regardless of nutrient availability (Bakker, 1959; Mirth & Riddiford, 2007;Nijhout, 2003;Robertson, 1963).Of these three size assessment check-points, critical weight is the most studied and best understood in *Drosophila*.

Once larvae reach their critical weight, environmental factors have a large impact on adult size. Larvae that starve before they achieve critical weight will delay their development until the nutrient supply improves. If nutrients are still abundant after larvae reach critical weight, they will continue to accumulate body mass (Mirth & Riddiford, 2007; Tennessen & Thummel, 2011). On the other hand, if postcritical weight larvae starve, they will stop growing in size. Since these starved larvae have reached their critical weight, they will enter metamorphosis within a similar time frame as fed larvae, but they will be smaller and will mature into smaller adults than the fed animals. This suggests that the mechanisms that regulate development and puparium formation must coordinate with nutrient utilization.

The endocrine cascade that follows critical weight achievement was originally described in the tobacco hookworm, *Manduca sexta* (Nijhout & Williams, 1974; Truman & Riddiford, 1974). Briefly, once larvae reach critical weight, juvenile hormone (JH) titers drop, causing a release of prothoracicotropic hormone, which signals to the prothoracic gland (PG) to produce ecdysone. However, this function of JH does not seem to be conserved in *Drosophila*, suggesting that critical weight is determined through another mechanism (Nijhout, 2003; Stern & Emlen, 1999).

Recent studies have elucidated some of the mechanisms required for critical weight assessment in *Drosophila*. One study showed that the *Drosophila* insulin receptor (InR), which has a conserved role in nutrition-dependent growth in animals, affects growth differently in precritical weight and postcritical weight larvae (Shingleton, Das, Vinicius, &

Stern, 2005). Before larvae reach critical weight, InR signaling influences developmental timing but not larval growth. In contrast, InR activity affects final body size but not developmental timing in postcritical weight larvae. This is consistent with the observations in starved larvae discussed above. Several other studies showed that in *Drosophila* the size of the PG affects developmental rate and body size (Caldwell, Walkiewicz, & Stern, 2005; Colombani et al., 2005; Mirth, Truman, & Riddiford, 2005). They did this by manipulating insulin-dependent growth in the PG. When PG growth was suppressed by the expression of PTEN, a phosphatase that antagonizes class I PI3K activity, dominant negative class I PI3K, or dominant negative Ras, the larvae were larger than controls and had a longer developmental period. Conversely, larvae with an enlarged PG due to either class I PI3K or Ras activation initiated metamorphosis earlier than controls and thus the adults were smaller. Interestingly, the effects of growth in the PG appear to be specific to the insulin signaling pathway and not to cell size increase in general. In the study done by Colombani et al., they increased PG size by manipulating two other growth pathways in addition to PI3K: Myc and cyclin D/Cdk4. Although activation of these two genes increased the size of the PG, they had no effect on pupal or adult size (Colombani et al., 2005).

It is clear from these studies that tissue growth coordinates with developmental timing through InR signaling; however, the signals that regulate this have not been well studied. Recently, two independent groups performed screens to identify molecules that couple tissue growth with developmental timing and identified a novel *Drosophila* insulin-like peptide (dilp), *dilp8* (Colombani, Andersen, & Léopold, 2012; Garelli, Gontijo, Miguela, Caparros, & Dominguez, 2012). Perturbing growth of larval imaginal disks through either damage or tumor promotion causes a delay in the time to pupariation, allowing the imaginal disks to reach their correct size (Menut et al., 2007; Poodry & Woods, 1990; Simpson, Berreur, & Berreur-Bonnenfant, 1980; Smith-Bolton, Worley, Kanda, & Hariharan, 2009). *dilp8* is highly induced in imaginal disks with growth perturbations (Colombani et al., 2012; Garelli et al., 2012). Importantly, knockdown of *dilp8* in tissues with abnormal growth prevents the delay in pupariation, suggesting that it is required for the coupling of tissue growth and developmental timing. Expression of *dilp8* in imaginal disks is also sufficient to delay the onset of metamorphosis, which can be overcome by feeding larvae ecdysone (Garelli et al., 2012). Additionally, coculture experiments reveal that ecdysone production in the ring gland is suppressed in response to Dilp8 produced by imaginal disks (Colombani et al., 2012). Taken together, these results suggest that Dilp8 is secreted by the imaginal disks and remotely acts on the ring gland to suppress ecdysone production and delay development. How Dilp8 suppresses ecdysone is not known, but it may signal through the InR pathway.

It has been shown that insulin signaling and ecdysone regulate each other antagonistically (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). A recent study has demonstrated a role for the nuclear cofactor, dDOR, in the relationship between insulin signaling and ecdysone. They show that dDOR is a coactivator of EcR, and that its expression is down-regulated by insulin signaling via the inhibition of FOXO activity (Francis, Zorzano, & Teleman, 2010). In addition, ecdysone induces translocation of dFOXO into the nucleus, promoting *dDOR* expression, which further activates EcR and initiates a feed-forward loop. Intriguingly, *dDOR* knockout flies have a salivary gland degradation defect, and DOR has been shown to regulate autophagy in both mammalian and *Drosophila* cells (Francis et al., 2010; Mauvezin et al., 2010). These results provide one of the few clues to how the relationship between insulin signaling, ecdysone, and autophagy functions (Fig. 4.3).

4. AUTOPHAGY AND DROSOPHILA DEVELOPMENT

Most autophagy studies have been done using either yeast or mammalian cell culture. While these studies have been essential to our understanding of the genetic mechanisms that regulate autophagy, there is little known about the impact of autophagy on the homeostasis of multicellular organisms. It would be interesting to understand how autophagy in different cell contexts, such as cell growth, cell survival, and cell death, affects the organism as a whole.

Drosophila is an ideal system for studying autophagy in a multicellular organism. The steroid and growth factor signaling pathways that regulate autophagy are similar in flies and humans. Importantly, *Atg* genes and their regulators are highly conserved between flies and humans (Baehrecke, 2003). In contrast to mammalian systems, *Drosophila* has little genetic redundancy and has single copies for most genes in the autophagic pathway and its regulatory pathways. In addition, autophagy is induced in *Drosophila* tissues in response to either nutrient starvation or the steroid hormone ecdysone (Lee & Baehrecke, 2001; Lee, Cooksey, & Baehrecke, 2002; Rusten et al., 2004).

4.1. Autophagy in growth and nutrient utilization

Autophagy is critical for proper nutrient utilization during *Drosophila* larval development. In the fly, the major storage site for glycogen, lipids, and proteins is the fat body, an organ that shares attributes with both mammalian adipose tissue and liver. The fat body provides an excellent model for studying the mechanisms that regulate autophagy. When larvae are deprived of amino acids, autophagy is induced in the fat body, and this starvation-induced autophagy is regulated by TOR signaling (Scott, Schuldiner, & Neufeld, 2004). It has been shown that inactivation of TOR signaling either by a *TOR* null mutant or by manipulating upstream regulators of TOR induces autophagy in the fat body of feeding larvae. On the other hand, activation of either TOR or class I PI3K suppresses starvation-induced autophagy in the fat body (Scott et al., 2004). These results, taken together with the result that constitutive expression of PI3K in the fat body causes reduced viability during starvation (Britton, Lockwood, Li, Cohen, & Edgar, 2002), suggest that proper regulation of the class I PI3K signaling pathway is necessary for autophagy to promote survival during starvation.

In addition to being necessary for survival during starvation, autophagy may have a critical role in lipid metabolism of the *Drosophila* fat body. In mammalian cells, it has been shown that there is a connection between autophagy and lipolysis as well as lipid storage. Singh et al. demonstrated that triglycerides (TGs) and lipid droplet (LD) proteins were associated with both autophagosomes and lysosomes. Moreover, inhibition of autophagy in mouse liver cells led to increased TGs and LDs *in vitro* and *in vivo*, while increased autophagy led to decreased TGs and LDs *in vitro* (Singh et al., 2009). Their data suggests that lipid accumulation during autophagy inhibition is a result of blocked lipolysis. By contrast, it has been shown that loss of either *Atg5* or *Atg7* in mouse adipocytes leads to reduced lipid accumulation and impaired adipocyte differentiation (Baerga, Zhang, Chen, Goldman, & Jin, 2009; Zhang et al., 2009). Similar results were obtained in a recent study of *Drosophila* larval fat body. *Atg7* loss-of-function mutants had smaller LDs in the fat body, indicating a lipid accumulation defect (Wang et al., 2012). One possible explanation for the discrepancies between these studies is that autophagy may affect lipid metabolism in a tissue-specific manner. It would be interesting to further investigate the relationship between autophagy and lipid metabolism and how it is regulated in different tissues.

Wang et al. (2012) provided insight into the relationship between lipid metabolism and autophagy. Members of the Rab small GTPase family have been associated with LDs, and

are known to participate in many cellular processes, including endocytosis, exocytosis, autophagosome formation, lysosome formation, and signaling transduction (Liu et al., 2007; Stenmark, 2009; Zehmer et al., 2009). In a screen for Rab proteins that affect LD size, Wang et al. found 18 Rab proteins that either increased or decreased LD size (Wang et al., 2012).They focused onRab32 and showed that as well as having smaller LDs, *Rab32* mutants have impaired autophagy in the fat body. Importantly, Rab32 localized on autophagosomes, but not LDs, suggesting that its effect on LD size is due to regulation of autophagy rather than a direct effect on LDs. Since different Rab proteins have different effects on LD size, investigating the remaining Rab proteins might shed some light on the regulation of the relationship between autophagy and lipid metabolism.

Autophagy is also induced in the fat body and other tissues, including the salivary glands and mid gut during development in response to rises in ecdysone titer. This developmental autophagy is induced during the wandering larval stage and metamorphosis at times when the animal is not feeding, suggesting that autophagy may play an important role in survival and even tissue growth during nonfeeding periods (Lee & Baehrecke, 2001; Lee, Cooksey, & Baehrecke, 2002; Rusten et al., 2004). In the fat body, programmed autophagy is induced in response to ecdysone late during the third larval stage. This induction requires the downregulation of class I PI3K signaling (Rusten et al., 2004), suggesting that regulation of the class I PI3K pathway is involved in both starvation-induced autophagy and developmental autophagy.

Studies in the *Drosophila* fat body have identified other genes that are necessary for autophagy induced in response to ecdysone. SNF4Aγ, the *Drosophila* homologue of the AMP-activated protein kinase (AMPK) γ sub-unit, was identified in a fat body screen for mutants that fail to induce autophagy in response to ecdysone (Lippai et al., 2008). AMPK is an evolutionarily conserved enzyme that maintains cellular energy balance and is an inhibitor of TOR signaling (Shaw, 2009). In mammalian cells, AMPK has been implicated in the induction of autophagy in response to stimuli other than starvation, including growth factor withdrawal and increased calcium signaling (Hoyer-Hansen et al., 2007; Liang et al., 2007). Importantly, several recent studies in mammalian cells have shown that AMPK may directly control ULK1, the mammalian homologue of Atg1, via phosphorylation; however, the exact sites of phosphorylation are still debated (Egan et al., 2011; Kim, Kundu, Viollet, & Guan, 2011; Lee, Park, Takahashi, & Wang, 2010; Shang et al., 2011). AMPK has also been shown to suppress cell proliferation in *Drosophila* (Mandal, Guptan, Owusu-Ansah, & Banerjee, 2005). Taken together, these studies suggest that AMPK is an important regulator of the relationship between autophagy and growth.

Studies in *Drosophila* have further investigated the relationship between autophagy and growth.TOR is a key regulator of cell growth that was first implicated in the regulation of autophagy when rapamycin, a TOR inhibitor, was shown to induce autophagy (Blommaart, Luiken, Blommaart, vanWoerkom, &Meijer, 1995). TOR represses autophagy through phosphorylation of Atg1 (Kamada et al., 2000; Scott, Juhász, & Neufeld, 2007). In *Drosophila* larval fat body, overexpression of Atg1 inhibits cell growth through a negative feedback mechanism on TOR. Conversely, *Atg1* mutant cells with reduced TOR signaling have increased growth (Scott et al., 2007). These results suggest that autophagy is a negative regulator of cell growth. Interestingly, it has been shown that inhibiting autophagy in a *TOR* null background enhances the *TOR* mutant phenotypes, including reduced growth rate, smaller cell size, and decreased survival (Scott et al., 2004).This suggests that under these conditions, in contradiction to its role as a negative regulator of growth, autophagy is necessary to promote cell survival and maintain growth.

The relationship between autophagy and growth signaling has also been studied in the context of degrading tissues during *Drosophila* metamorphosis. Growth arrest is required for the induction of autophagy in degrading salivary glands (Berry & Baehrecke, 2007). This growth arrest is regulated by the class I PI3K pathway. Maintaining growth in the salivary glands through expression of activated Ras, Akt, or the class I PI3K catalytic subunit Dp110, inhibits autophagy and gland degradation. In addition, coexpression of a dominant negative TOR with either Ras or Dp110 partially suppresses the overgrowth phenotypes and the salivary gland degradation defects (Berry & Baehrecke, 2007). These data suggest that cell growth regulators signal through TOR to inhibit autophagy and prevent salivary gland degradation. Further, overexpression of Atg1, which induces autophagy, suppresses the Dp110 persistent salivary gland phenotype, while *Atg* loss-of-function mutations cause persistent salivary glands (Berry & Baehrecke, 2007), indicating that both growth arrest and autophagy are required for proper salivary gland degradation.

A recent study has observed a similar relationship between growth arrest and autophagy during midgut programmed cell death in *Drosophila*. In the midgut, as in the salivary glands, growth arrest occurs before programmed cell death induction (Denton, Chang, et al., 2012). When cell growth in the midgut is maintained by expression of either activated Ras or Dp110, autophagy is suppressed and midgut degradation is delayed (Denton, Chang, et al., 2012). These results indicate a role for growth arrest in midgut programmed cell death. In contrast, inhibition of growth by the expression of PTEN or TSC1/TSC2, negative regulators of class I PI3K signaling, results in smaller midguts and premature autophagy induction. This growth inhibition can be suppressed by knockdown of either *Atg1* or *Atg18* in a PTEN or TSC1/TSC2 expressing background (Denton, Chang, et al., 2012). Interestingly, knockdown of *Atg* genes alone in the midgut causes persistent PI3K growth signaling and a significant delay in midgut degradation. These results suggest that in the midgut, growth and autophagy have a reciprocal relationship as in the salivary glands; however, there is also a feedback mechanism by which autophagy downregulates class I PI3K signaling. The nature of this feedback mechanism is unknown and deserves future investigation.

There has been some recent progress on the study of how cell growth arrest is regulated in dying salivary glands. The evolutionarily conserved Warts (Wts)/Hippo (Hpo) signaling pathway is an important negative regulator of cell growth that functions through the inactivation of Yorkie (Yki), a transcriptional coactivator and positive regulator of growth (Huang, Wu, Barrera, Matthews, & Pan, 2005). Loss-of-function mutations in the Wts pathway or overexpression of Yki lead to tissue overgrowth (Huang et al., 2005). Importantly, *wts* is required for growth arrest and autophagy induction in degrading salivary glands (Dutta & Baehrecke, 2008). Disruption of this pathway by mutations in *wts* and *hpo* or knockdown of *sav* and *mats* prevents salivary gland degradation (Dutta & Baehrecke, 2008). Surprisingly, overexpression of Yki fails to inhibit salivary gland degradation, suggesting that Wts regulates salivary gland growth in a Yki-independent manner. Significantly, *wts* mutants cause persistent class I PI3K signaling in salivary glands, and knockdown of *chico* or expression of dominant-negative TOR suppresses the *wts* cell death defects (Dutta & Baehrecke, 2008). These data suggest that Wts regulates salivary gland cell growth in a class I PI3K-dependent manner. However, Wts does not have a common role in programmed cell death. Despite the clear requirement for class I PI3K signaling in the regulation of cell growth and cell death in the midgut, knockdown of *wts* does not affect midgut morphology or degradation (Denton, Chang, et al., 2012).

4.2. Autophagy and cell death

Programmed cell death is a highly conserved and genetically regulated fundamental biological process. During development, cell death is required for tissue pattern formation and to maintain tissue homeostasis. Cell death also functions to remove abnormal or damaged cells. Schweichel and Merker (1973) described three major types of cell death during mammalian development based on morphology and involvement of the lysosomal compartment. Type I cell death, or apoptosis, is characterized by caspase activation, cell shrinkage, cytoplasmic blebbing, nuclear and DNA fragmentation, and engulfment by a phagocyte where the lysosome of the engulfing cell degrades the dying cell (Kerr, Wyllie, & Currie, 1972). In contrast to apoptosis, type II cell death, or autophagic cell death, requires little or no help from phagocytes, and the dying cell is degraded by its own lysosome. Type III cell death, or necrosis, is the least common form of cell death, and it has no known lysosomal involvement.

Type II cell death is observed in a variety of organisms. The plant, *Arabidopsis*, requires type II cell death for the formation of tracheary elements (Kwon et al., 2010). Type II cell death has also been observed in several tissues during mammalian development, including regression of the corpus luteum and involution of mammary and prostate glands (Clarke, 1990). Type II cell death is best characterized in insects and has been observed in several tissues during development, including dying flight muscles of the Hawkmoth *Manduca sexta* (Lockshin & Williams, 1965), and degrading salivary glands and midgut in *Drosophila* (Lee & Baehrecke, 2001; Lee, Cooksey, & Baehrecke, 2002). Although autophagosomes are present in dying cells with type II morphology, the role of autophagy in cell death remains controversial (Denton, Nicolson, et al., 2012; Levine&Yuan, 2005).

Studies of dying larval tissues during *Drosophila* metamorphosis have provided evidence for a role of autophagy in programmed cell death. As described above, a peak in ecdysone titer triggers salivary gland degradation during metamorphosis. Several *Atg* genes exhibit increased transcription in salivary glands in response to the rise in ecdysone, including *Atg2, Atg3, Atg4, Atg5, Atg7*, and *Atg18* (Gorski et al., 2003; Lee et al., 2003). Additionally, mutations in transcription factors downstream of the EcR inhibit transcription of *Atg*-related genes and prevent proper salivary gland cell death (Lee et al., 2003), suggesting that ecdysone-induced autophagy promotes cell death. It was not until recently though that the function of autophagy in cell death was rigorously tested *in vivo*. Mutations in *Atg8, Atg18, Atg2*, or *Atg3* or decreased function of *Atg1* all result in incomplete degradation of the larval salivary glands (Berry & Baehrecke, 2007). In addition, knockdown of *Atg3, Atg6, Atg7*, or *Atg12* specifically in the salivary glands leads to incomplete gland destruction, suggesting that autophagy functions in a tissue-autonomous manner in these dying cells (Berry & Baehrecke, 2007). Moreover, misexpression of Atg1 in the salivary glands induces autophagy and leads to premature gland degradation in a caspase-independent manner (Berry & Baehrecke, 2007). This is in contrast to previous work which showed that overexpression of Atg1 in the fat body induces cell death that depends on caspase function (Scott et al., 2007).

There is also mounting evidence for a role of autophagy during programmed cell death of the larval midgut. Similar to salivary glands, larval midgut destruction is triggered by a peak in ecdysone titer at the end of larval development. The dying midguts have increased autophagosome formation, and inhibition of autophagy by loss-of-function mutations in *Atg2* or *Atg18* or knockdown of either *Atg1* or *Atg18* severely delays midgut removal (Denton et al., 2009). Additionally, overexpression of Atg1 in the larval midgut is sufficient to induce autophagy and premature degradation (Denton, Chang, et al., 2012). Surprisingly, caspases are active, but they are not required for removal of the midgut (Denton et al., 2009;

Denton, Shravage, Simin, Baehrecke, & Kumar, 2010), indicating that there is a complex relationship between autophagy and caspases in this tissue.

Autophagy and caspases have a complex relationship that may be context dependent. During salivary gland degradation, the rise in ecdysone titer triggers increased transcription of not only *Atg* genes but also the proapoptotic genes, *rpr* and *hid*, caspases, the BCL-2 family member *buffy*, and *ark*, the fly *Apaf-1* homologue (Dorstyn, Colussi, Quinn, Richardson, & Kumar, 1999; Jiang, Baehrecke, & Thummel, 1997; Lee, Simon, Woodard, & Baehrecke, 2002). Caspase activation occurs in the glands, but expression of the caspase inhibitor p35 only partially inhibits salivary gland degradation (Lee & Baehrecke, 2001). Additionally, *ark* mutants have a partial salivary gland degradation defect, but autophagy occurs normally, suggesting that *ark* may function downstream or parallel to autophagy in programmed cell death (Akdemir et al., 2006; Mills et al., 2006). Significantly, inhibiting both caspases and autophagy by expressing p35 in salivary glands of *Atg18* loss-of-function mutants or with dominant negative Atg1 results in increased persistence of the salivary glands (Berry & Baehrecke, 2007). These results suggest that autophagy and caspases function in parallel during salivary gland cell death. Many of the components of the apoptotic machinery are also upregulated in dying midguts. Despite the presence of high levels of caspase activity, p35 expression or genetic ablation of the canonical caspase activation pathway has no effect on midgut degradation (Denton et al., 2009). This is in contrast to what has been observed in salivary glands, and it would be interesting to study what causes these distinct differences between how programmed cell death is executed in these two tissues.

Although these *in vivo* studies indicate a role for autophagy in programmed cell death, the mechanistic differences that determine whether autophagy will support cell survival or cell death are not clear. Recently, Draper (Drpr), the *Drosophila* homologue of *Caenorhabditis elegans* engulfment receptor CED-1, and other components of the engulfment pathway were shown to be required for induction of autophagy during cell death (McPhee, Logan, Freeman, & Baehrecke, 2010). Null mutations in *drpr* and salivary gland-specific knockdown of *drpr* prevent induction of autophagy and cause persistent salivary glands. Expression of Atg1 in *drpr* mutants is sufficient to rescue the salivary gland degradation defect, indicating that Drpr functions upstream of autophagy. Surprisingly, clonal analysis of degrading glands reveals that Draper functions in a cell-autonomous manner, as there is only a reduction of autophagy in the *drpr* mutant cells. Interestingly, knockdown of *drpr* in the fat body does not affect starvation-induced autophagy, implicating *drpr* as the first known factor to regulate autophagy's role in cell death but not cell survival (McPhee et al., 2010). It would be interesting to further investigate how Drpr is regulated in salivary glands and why an engulfment receptor is functioning cell-autonomously.

5. CONCLUSIONS

Organisms require a balance between cell survival and cell death to maintain homeostasis, and although *in vivo* evidence supports a role for autophagy in both cell survival and cell death, many fundamental questions remain. Since autophagy is involved in both protecting and killing the cell, it is important to determine the mechanisms that decide between these cell fates. One possibility is that autophagy selectively depletes a cell survival factor or an essential organelle, which leads to cell death (Abeliovich, 2007; Nezis et al., 2010; Yu et al., 2006). Another possibility is that there is a threshold of autophagic flux that is crossed to promote cell death. Extended growth factor withdrawal in apoptotic-resistant mouse cells leads to stress-induced autophagy and eventual death by depletion of cellular resources (Lum et al., 2005). Under more physiological conditions, degradation of the *Drosophila* salivary glands and midgut is preceded by an increase in both transcription of the *Atg* genes and autophagosome levels. Additionally, mis-expression of Atg1 in several tissues promotes

cell demise, supporting the idea that excessive autophagy leads to cell death. However, excessive autophagy might not always be enough to kill, and other death factors may be required in addition to autophagy. Cell death induced by Atg1 mis-expression in the fat body is caspase dependent. Further, salivary glands require caspases and autophagy, functioning in parallel, to fully degrade (Berry & Baehrecke, 2007).

Autophagy has been shown to be both an alternative form of cell death in nonphysiological conditions and a necessary component of cell death in physiological contexts; however, why cells die by autophagy is not understood. Apoptosis requires a phagocyte to engulf the dying cell, while autophagic cell death has little or no phagocyte involvement. One possibility is that phagocytes have restricted access to the dying cells. In *Drosophila*, the adult midgut forms around the degrading larval midgut isolating the dying cells from the rest of the tissues. Similarly, *in vitro* models of mammary lumen formation, where the dying cells are isolated from phagocytes, implicate the necessity of both caspases and autophagy for elimination of the dying cells (Debnath et al., 2002; Mills, Reginato, Debnath, Queenan, & Brugge, 2004). Alternatively, large cells and tissues, such as the giant larval salivary glands, may be too big to degrade by phagocytosis alone, and they require autophagy for the bulk degradation of their cytoplasm. Finally, autophagy may contribute to nutrient resource reallocation and survival in multicellular organisms. In yeast and mammalian cell culture, autophagy degrades cellular content to produce ATP and resources to protect the cell during starvation. Interestingly, autophagic cell death of tissues in *Drosophila* occurs during a time when the animal receives no external nutrients and must rely on its nutrient stores for survival and development of adult structures. Further, the majority of *Atg* mutants are pupal lethal, suggesting that autophagy is necessary to survive metamorphosis. Thus, although autophagy is killing individual cells and tissues, this form of cell death could be promoting organism survival. Future studies in *Drosophila* will hopefully lead to a better understanding of autophagy's dual roles in life and death.

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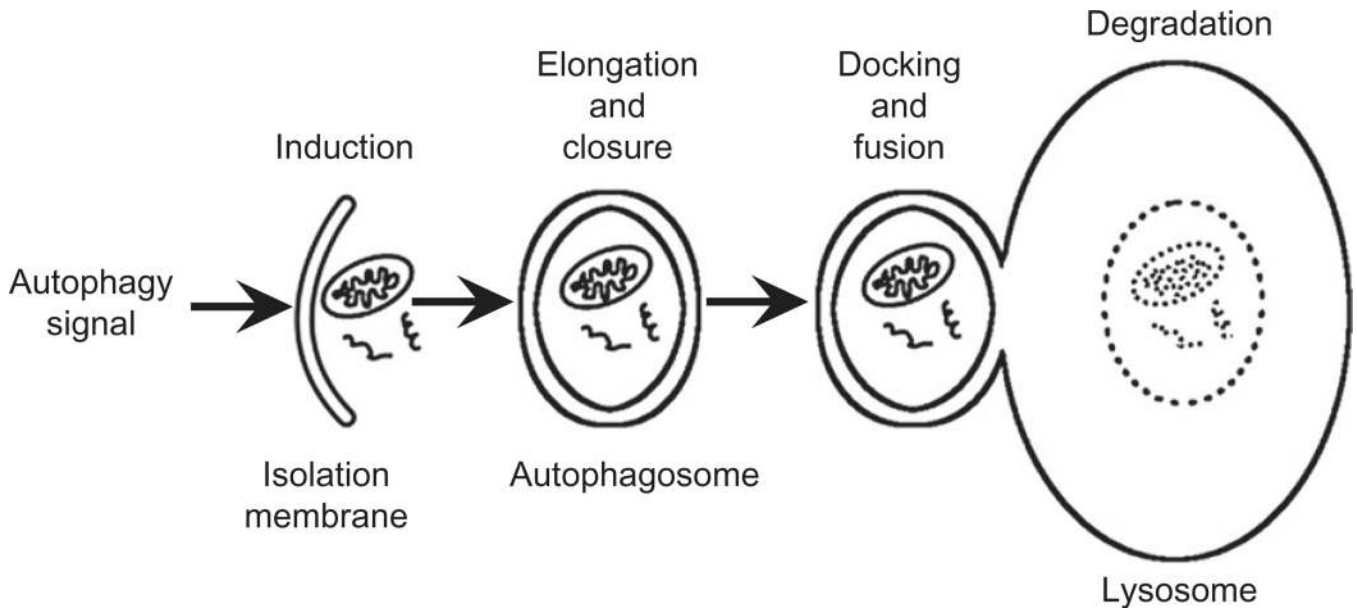

Figure 4.1.
Diagram of the steps in autophagy. Autophagy is a catabolic process by which organelles and cytoplasmic proteins are degraded. Induction of autophagy results in the formation of an isolation membrane, which expands and closes around cytoplasmic material, forming the double-membraned autophagosome. The autophagosome traffics to the lysosome where it docks and fuses, releasing its inner membrane and its contents. The autophagosome contents are degraded by lysosomal enzymes and are recycled back to the cytoplasm through permeases.

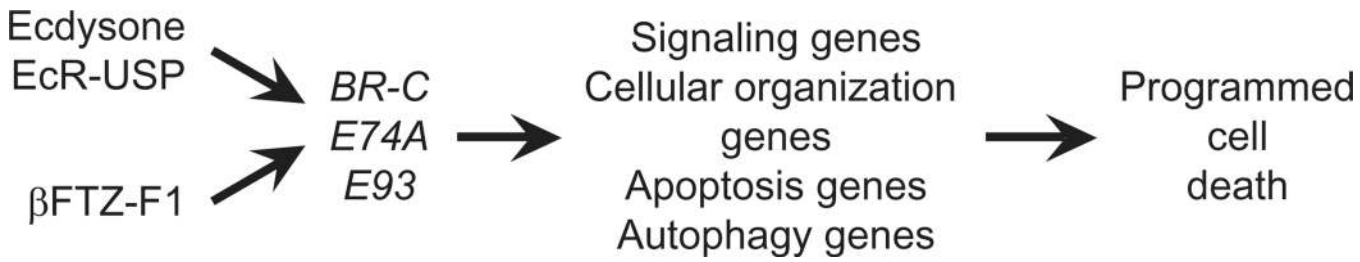

Figure 4.2.

Genetic regulation of ecdysone-induced autophagy in *Drosophila* salivary glands. At 10 h after puparium formation, there is a rise in ecdysone titer, and ecdysone binds to its heterodimeric receptor which consists of EcR and USP. The ecdysone receptor complex functions together with βFTZ-F1 to induce transcription of the early genes; *BR-C*, *E74A*, and *E93*. The early genes activate transcription of many late genes involved in signaling, cellular organization, apoptosis, and autophagy.

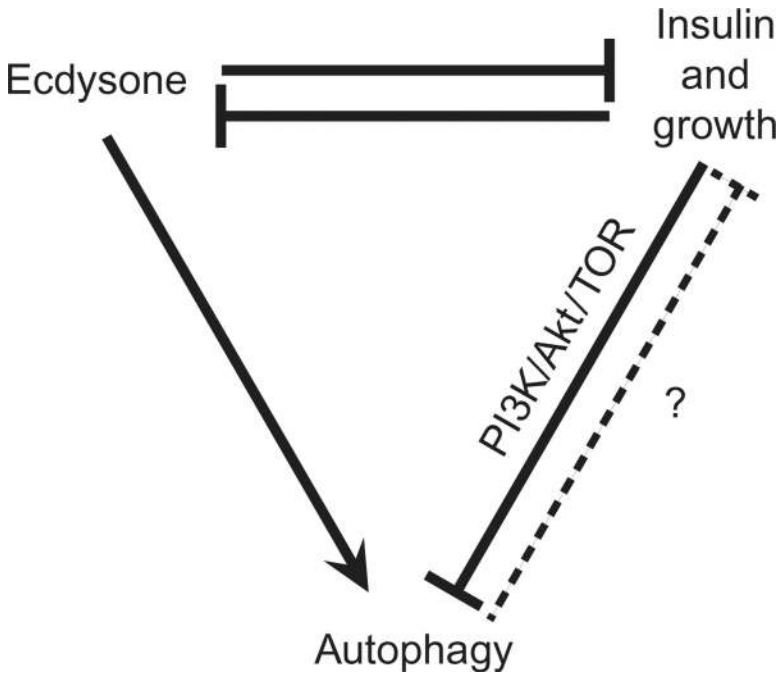

Figure 4.3.
Relationship between ecdysone, autophagy, and insulin signaling and growth. Ecdysone and insulin have opposing effects on autophagy; ecdysone induces autophagy, and insulin inhibits autophagy through class I PI3K and TOR signaling. Ecdysone and insulin also antagonize each other, suggesting that a balance between these two hormones may be required to regulate autophagy. For example, increased ecdysone would inhibit insulin signaling, releasing insulin's inhibition on autophagy and further inducing autophagy. Whether autophagy itself regulates ecdysone and insulin is unclear; however, in some contexts autophagy may be a negative regulator of growth.