Biochimica et Biophysica Acta 1793 (2009) 1452–1460

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Review

Autophagy in *Drosophila melanogaster*

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ARTICLE INFO

Article history:
Received 1 December 2008
Received in revised form 18 February 2009
Accepted 19 February 2009
Available online 2 March 2009

Keywords:
Drosophila
Autophagy
Cell survival
Cell growth
Cell death

ABSTRACT

Macroautophagy (autophagy) is a bulk cytoplasmic degradation process that is conserved from yeast to mammals. Autophagy is an important cellular response to starvation and stress, and plays critical roles in development, cell death, aging, immunity, and cancer. The fruit fly *Drosophila melanogaster* provides an excellent model system to study autophagy *in vivo*, in the context of a developing organism. Autophagy (*atg*) genes and their regulators are conserved in *Drosophila*, and autophagy is induced in response to nutrient starvation and hormones during development. In this review we provide an overview of how *Drosophila* research has contributed to our understanding of the role and regulation of autophagy in cell survival, growth, nutrient utilization, and cell death. Recent *Drosophila* research has also provided important mechanistic information about the role of autophagy in protein aggregation disorders, neurodegeneration, aging, and innate immunity. Differences in the role of autophagy in specific contexts and/or cell types suggest that there may be cell-context-specific regulators of autophagy, and studies in *Drosophila* are well-suited to yield discoveries about this specificity.

1. Introduction

Autophagy is a catabolic process that is ubiquitously implemented by eukaryotic cells. Three general types of autophagy have been described, macroautophagy, microautophagy, and chaperone-mediated autophagy [1, 2]. Of the three types, macroautophagy, a largely non-selective degradation process to eliminate macromolecules and organelles, has been best characterized and will hereby be referred to as autophagy. Whereas the Ubiquitin Proteasome System (UPS) is used to degrade short-lived proteins specifically targeted for degradation, autophagy is generally thought to be used for the bulk degradation of long-lived proteins. During autophagy, cytosolic components are sequestered by a double-membrane vesicle, the autophagosome, which delivers cargo to the lysosome for recycling.

Autophagy is an important cellular response to stress and a survival mechanism during starvation that is conserved in yeast, worms, flies, and mammals [3, 4]. In response to starvation, autophagy functions in the production of amino acids, providing the building blocks for new protein synthesis. Autophagy is also a mechanism for the production of mitochondrial substrates to produce the energy required to survive starvation [5]. In addition, autophagy is important for the elimination of damaged/unwanted organelles and protein aggregates [2, 6]. In animal cells, autophagy also plays a role in cellular remodeling during development and differentiation, and in the elimination of invasive microorganisms. Alterations and deficiencies in autophagy (*atg*) genes are associated with developmental and cell death defects, sensitivity to starvation and stress, decreased longevity, neurodegeneration, and tumor progression [2, 6–8].

The pathways that regulate autophagy are evolutionarily conserved [9, 10]. The protein kinase Target of Rapamycin (TOR) plays a central role in nutrient sensing and autophagy regulation. When nutrients are readily available, TOR is activated through the Class I phosphatidylinositol-3-kinase (PI3K) signaling pathway, and TOR inhibits autophagy through direct phosphorylation and repression of Atg1 [11, 12]. When nutrients are scarce, TOR becomes inactivated, its repression of Atg1 is relieved, and autophagy is induced. In yeast, autophagy induction requires Atg1, Atg13, and Atg17, and TOR has been shown to regulate the association of Atg1 with Atg13 via Atg13 phosphorylation [12, 13]. Nucleation of the autophagic membrane requires phosphatidylinositol phosphorylation, which in yeast requires the formation of a complex that includes Atg6/Beclin1, Vps34/Class III PI3K, Atg14 and Vps15 [14–16]. In mammalian cells, additional regulators of the Beclin1/Vps34 complex have been identified, including Ambra1, UVRAG and Bif-1, and the mammalian Atg14 homolog, Barkor [17–20]. In addition, 2 distinct Beclin-1 complexes have been described in yeast and mammalian cells that may play distinct roles in membrane trafficking pathways [15, 16, 21].

Little is known about the origin of the autophagic membrane, and this is a subject of debate [22]. In yeast, autophagy proteins gather at a punctate structure, the Pre-Autophagosomal Structure (PAS), near the vacuole (the yeast lysosome) [4]. A recent study suggests that in mammalian cells, nucleation of the autophagic membrane begins in a punctate compartment that is in dynamic equilibrium with the Endoplasmic Reticulum (ER) [23]. This work is consistent with a previous study suggesting that ER membranes are used to form

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doi:10.1016/j.bbamcr.2009.02.009

autophagosomes based on the localization of ER proteins in autophagosomal membranes [24].

Two ubiquitin-like conjugation systems, conserved from yeast to mammals, function in autophagosome formation [25, 26]. The formation of an Atg12-5-16 complex on the isolation membrane requires the E1-like activating enzyme Atg7 and the E2-like conjugating enzyme Atg10 [27–31]. This complex disassociates upon formation of the autophagosome. The second conjugation system requires the activity of Atg7 and the E2-like conjugating enzyme Atg3 [29, 32–34]. In this step, cytosolic Atg8 (LC3 in mammals) is modified by the attachment of the phospholipid anchor phosphatidyl-ethanolamine (PE) following its cleavage by the cysteine protease Atg4 [35–37]. This step results in the localization of Atg8-PE to the isolation membrane, and has been proposed to contribute to elongation of the autophagic membrane [38]. Atg8 remains associated with the autophagosome until it is trafficked to the lysosome, when the outer membrane of the autophagosome fuses with the lysosome to form the autolysosome, and Atg4 subsequently releases Atg8 from PE [35]. For these reasons, Atg8/LC3 is a widely used marker of autophagosomes [39]. Upon autophagosome formation, the inner membrane of the autophagosome and its contents are degraded by lysosomal hydrolases, and nutrients are subsequently released into the cytosol for recycling [3].

2. Autophagy in *Drosophila* development

Pioneering genetic screens in the yeast *Saccharomyces cerevisiae* advanced our understanding of autophagy by identifying the genes that are required for this catabolic process [40–43]. The complexity of multicellular animals presents several interesting questions about autophagy and its relationship to nutrient utilization, cell growth, cell survival and cell death. For example, the ability of animals to respond to nutrient deprivation and adjust metabolic and catabolic processes to maintain homeostasis suggests that the mechanisms that regulate autophagy may differ under specific cellular contexts.

Drosophila melanogaster is an excellent genetic model for higher animals. *Drosophila* has a short life cycle, a wide variety of genetic tools available, and mutants and RNAi lines have been systematically generated (http://flybase.org). *atg* genes and their regulators are highly conserved in *Drosophila* [9, 44], and in many cases, in contrast to mammalian systems, single orthologs to *atg* genes exist in *Drosophila*, allowing for non-redundant genetic studies. Autophagy is induced in many *Drosophila* tissues in response to nutrient restriction; for example, in the fat body of starving larvae, and during the pupal stage upon the cessation of feeding. In addition, autophagy is induced in *Drosophila* in response to the steroid hormone 20-hydroxyecdysone (ecdysone) in both the fat body [45] and in dying larval structures such as the intestine and salivary glands [46, 47]. Thus, *Drosophila* serves as an excellent model to study autophagy *in vivo*, in response to nutrient restriction and also in the context of programmed cell death that occurs during development.

Drosophila atg gene mutant phenotypes suggest a role for autophagy in development [9, 48]. Null mutations in *atg1* are pupal lethal [49]. Surprisingly, however, mutations in some *atg* genes essential for autophagy are not lethal, even though these flies appear to possess greatly attenuated autophagy. Null *atg7* mutants develop normally whereas strong *atg8* hypomorphic mutations are semi-lethal [11, 50–52]. Both *atg7* and *atg8* mutants are hypersensitive to starvation and oxidative stress, exhibit degenerative neuronal defects, accumulate ubiquitin-positive aggregates in neurons, and have a shortened lifespan [11, 50–52]. This range in phenotypes suggests that some autophagy genes could play specialized roles, while others may be more pleiotropic and should be investigated for phenotypes that are not related to autophagy. The fact that *atg7* null mutations are not lethal suggests a few different possibilities. Some *atg* genes may function redundantly, or it could be that other mechanisms may compensate for macroautophagy deficiencies during *Drosophila* development, such as chaperone-mediated autophagy or microautophagy [2]. Alternatively, given that certain autophagy gene mutations have cell-context-specific effects, there could be factors that determine specificity. Finding such factors will require studies to be carried out in nutritional contexts that are relevant to physiology and development, rather than in cell lines, and *Drosophila* is well-suited for this type of study.

3. Autophagy in growth and nutrient utilization

To develop to the proper size, animals require the coordination of cell growth, division and death within individual tissues, and this is influenced by environmental factors including nutrient availability [53]. *Drosophila* development provides a useful system to investigate the relationship between nutrients, autophagy, growth and development. Fly development lasts approximately 8 days in the laboratory; embryogenesis is 1 day long, the 3 larval feeding stages combine to last 3.5 days, and the transformation to an adult, known as metamorphosis, is 3.5 days long. Like vertebrate animals, fly development is controlled by growth factors and hormones. Insulin and insulin-like growth factors are secreted when animals are feeding, stimulating protein synthesis, cell growth, and the processes that are required for animal growth [54]. In addition, the steroid ecdysone defines the length of the developmental period in *Drosophila*, as peaks in this steroid regulate larval molting and metamorphosis [55]. Several recent studies have investigated the relationship between growth factor and ecdysone signaling in flies [56, 57]. Surprisingly, few studies have investigated the relationship between these signaling pathways, autophagy and animal development.

Autophagy plays an important role in nutrient utilization during *Drosophila* larval development, and studies in the *Drosophila* fat body under conditions of starvation have provided important insights into the mechanisms that regulate autophagy. The fly fat body is a nutrient storage and mobilization organ akin to the mammalian liver and autophagy is induced in the fat body in response to amino acid starvation [49]. Either null mutations in *TOR*, or repression of TOR by modulation of components of the class I PI3K pathway, lead to the induction of autophagy in the fat body of feeding larvae, and result in the inhibition of growth, a reduction in cell size, and decreased viability [49]. Conversely, expression of either activated TOR, or activation of components of the PI3K signaling pathway, suppresses starvation-induced autophagy [49]. However, unlike in mammalian cells, the suppression of starvation-induced autophagy in this context does not require S6K phosphorylation [49].

Autophagy is also induced in several *Drosophila* tissues as a normal physiological response to the rise in hormones during metamorphosis. Autophagy and multiple *atg* gene RNA levels are induced following rises in ecdysone that trigger cell death of the larval midgut and salivary glands during metamorphosis [46, 47, 58], suggesting that autophagy, and possibly cell death, play important roles in the maintenance of nutrient homeostasis when developing animals are not feeding. In the fat body, Class I PI3K pathway signaling is down-regulated, and autophagy is induced, following the rise in ecdysone at the end of the third larval stage that triggers metamorphosis [45], indicating that regulation of the PI3K pathway is involved in the induction of autophagy in response to ecdysone as well as starvation. A recent screen for mutants that fail to induce autophagy in the fat body in response to ecdysone identified the *Drosophila* homologue of the AMP-activated protein kinase (AMPK) γ subunit, SNF4Aγ [59]. AMPK has been implicated in the regulation of autophagy and cell survival following growth factor withdrawal in mammalian cells [60]. In addition, a recent study indicates that AMPK represses TOR and induces autophagy under nutrient-rich conditions in response to calcium signaling in mammals [61]. Therefore, these studies suggest a conserved role for AMPK in the regulation of TOR and autophagy.

Studies in the *Drosophila* fat body have also shown that mutations in genes required for endosomal biogenesis are required for autophagy [62]. Mutations in *escrt* genes encoding the ESCRT proteins I, II, and III, as well as mutations in *vps4*, the ESCRT complex regulatory ATPase, all lead to an accumulation of autophagosomes in the fat bodies of fed larvae [62, 63]. Furthermore, in *escrt-II* mutant cells, lysosomal associated membrane protein-1 (LAMP-1)-positive structures are distinct from Atg8-positive structures, suggesting a failure in the fusion of autophagosomes and lysosomes. This study also showed that mutations in *fab1*, an endosomal PI3 5-kinase, lead to the accumulation of autolysosomes that fail to degrade their contents, suggesting that *fab1* is required for the maturation, and possibly the acidification, of autophagosomes [62].

The Vps34/Class III PI3K complex is a critical and conserved regulator of autophagy. This complex has been implicated in the regulation of endosomal maturation/lysosomal biogenesis as well as autophagy. It is possible that endosomal maturation/lysosomal biogenesis and autophagy may be co-regulated, although it is also possible that distinct Vps34 complexes regulate these vesicular trafficking pathways. Studies of mammalian cells suggest that amino acids stimulate TOR by activating Vps34/Class III PI3K [64, 65]. However, some differences in the regulation of autophagy may exist between insect and mammalian systems. In *Drosophila*, whereas *vps34* is required for starvation-induced autophagy in the larval fat body, null mutations in *vps34* do not affect TOR signaling [66]. It is not clear whether the differences between these results in flies and mammalian cells reflect evolutionary differences, or possibly cell-context-specific differences between *in vitro* and *in vivo* studies.

Studies in *Drosophila* have also provided new insights into the relationship between autophagy and growth. Rapamycin treatment induces autophagy [67], and this revealed the critical regulatory role of TOR on autophagy that has been noted in many studies [10, 54, 68]. TOR represses autophagy through phosphorylation of Atg1 [11, 12]. Scott et al. [11] demonstrated that in the *Drosophila* larval fat body, over-expression of Atg1 inhibits cell growth, and this involves a negative feedback mechanism on TOR. In addition, *atg1* mutant cells have a growth advantage when TOR signaling is reduced [11]. Thus, there is a reciprocal relationship between autophagy and cell growth. Interestingly, *atg* gene disruption in *Drosophila tor* mutant fat body cells does not restore growth, but enhances the cell size reduction in *tor* mutants, suggesting that in this context, autophagy is required to supply nutrients in order for cells to grow [49]. Similarly, null *atg5* or *atg7* mutant mice lack the energy required to survive post-natal nutrient depletion [69, 70].

A recent study in mammalian cells, as well as in *Drosophila*, provided additional mechanistic information regarding the regulation of cell growth and autophagy. Kim et al. [71] found that Rag GTPases activate TOR in response to amino acid signals, and this activation is in parallel to Rheb signaling. Further, expression of constitutively active Rag GTPases in the absence of amino acids activates TOR, suppresses autophagy, and leads to an increase in organ size, whereas expression of dominant negative Rag GTPases leads to a decrease in organ size. Therefore, the reciprocal relationship between autophagy and cell growth involves the activation of Rag GTPases, and this mechanism is conserved [71, 72].

Autophagy and growth also exhibit a reciprocal relationship in the context of autophagy that occurs during cell death in *Drosophila* larval salivary glands. Growth arrest via regulation of the PI3K pathway is required for the induction of autophagy that occurs during salivary gland degradation [73]. Activation of positive regulators of growth, including Ras, Akt, or the PI3K catalytic subunit Dp110, inhibits autophagy and degradation of salivary glands [73]. Coexpression of the caspase inhibitor p35 with Dp110 enhances the Dp110-induced partial salivary gland degradation phenotype, suggesting that caspases function in parallel to growth arrest in this context. Importantly, expression of Atg1 and activation of autophagy suppress the persistent phenotype in Dp110-expressing salivary glands, and *atg* loss of function mutations prevent the destruction of salivary glands [73]. Thus, growth arrest via down-regulation of Class I PI3K is required for the induction of autophagy and salivary gland degradation. Further, the inhibition of salivary gland degradation by positive regulators of cell growth requires the activity of TOR, suggesting that the inhibition of autophagy by cell growth regulators takes place through TOR signaling [73].

Recent work has elucidated how cell growth arrest is regulated in dying salivary glands. Loss-of-function mutations in the Warts/Hippo pathway, or over-expression of Wts downstream target Yorkie (Yki), the ortholog of mammalian Yes-associated protein (Yap), lead to tissue overgrowth [74]. However, it is not known how these genes influence cell growth and whether this occurs through signaling to the class I PI3K pathway. Significantly, *warts* (*wts*) is required for growth arrest and the induction of autophagy in dying salivary glands [75]. Mutations in *wts*, as well as knockdown of *wts* pathway components *hpo*, *sav* and *mats*, prevent salivary gland degradation. Surprisingly, however, over-expression of Yki in salivary glands does not phenocopy *wts* mutations, suggesting that in salivary glands, Wts signals independently of Yki. Significantly, *wts* mutants have altered class I PI3K markers and require the function of TOR and the insulin receptor substrate *chico* to inhibit salivary gland degradation [75]. Therefore, Wts influences PI3K signaling in salivary glands. These data provide mechanistic information about how cell growth is regulated during salivary gland cell death. It will be interesting to see whether Wts regulates cell growth in a PI3K-dependent manner in other systems.

4. Autophagy and cell death

Programmed cell death is a conserved and genetically regulated process that plays important roles throughout the lives of metazoans [76]. Cell death functions in the formation and shaping of structures during development, and in tissue homeostasis during adulthood. In 1973, Schweichel and Merker described three prominent types of programmed cell death that occur in developing mammalian cells and are distinguished by dying cell morphology and use of the lysosome [77, 78]. Type I cell death, or apoptosis, occurs in cells that die in isolation from one another, and is characterized by the condensation of nuclei and cytoplasm, DNA fragmentation, and phagocytosis by a secondary cell where the lysosome of the engulfing cell degrades the dead cell [79]. In type II, or autophagic cell death, cells die in groups, and cell destruction occurs with little or no phagocytosis. Type II cell death is characterized by the presence of autophagosomes that degrade cytoplasmic contents of the dying cell. The third and least common type, non-lysosomal cell death, is characterized by the lack of known lysosomal activity in the dying cell.

Type II cell death is observed at several stages during mammalian development, including regression of the corpeus luteum, involution of mammary and prostate glands, and regression of Mullerian duct structures during male genital development [78]. Type II cell death has also been observed during insect development, for example in dying flight muscle cells of the Hawkmoth *Manduca sexta* after which the term programmed cell death was originally coined [76]. *Drosophila* larval salivary glands that die during metamorphosis also exhibit Type II morphology; autophagosomes are present in the cytoplasm, and phagocytosis is not observed [46, 80]. While the presence of autophagosomes in cells that die by type II morphology has been well documented, the role of autophagy in cell death is controversial [81, 82].

Several studies demonstrate a role for autophagy in mammalian development [83]. *atg5*, as well as *atg7*, null mutant mice exhibit few developmental defects at birth, but die within 1 day of delivery, and when un-suckled, die earlier than wild-type mice [69, 70]. Therefore in this context, autophagy is required to generate the energy necessary to survive nutrient deficiency. Autophagy may also play a

role in the context of cell death that occurs during mammalian development. A study utilizing an *in vitro* mouse stem cell model of embryogenesis demonstrated that null *atg* mutant embryonic stem cells are not deficient in the activation of programmed cell death, but fail to generate and display signals that are required for phagocytosis of dying cells [84]. Significantly, addition of methylpyruvate was sufficient to rescue *atg* mutant engulfment and clearance defects, indicating that the role of autophagy is to provide the metabolic substrates needed to complete this process. A subsequent study reported similar findings in the context of cell death that occurs in developing chick retina [85]. When autophagy was inhibited during retinal development, apoptotic cells accumulated, and this accumulation correlated with a deficiency in phosphatidylserine exposure [85]. Thus, these studies suggest that in the context of cell death that occurs during mammalian development, autophagy deficiency is associated with a failure in phagocytic clearance of apoptotic cells. Beyond these studies, little is known about the role of autophagy in developmental cell death in mammalian systems, and the cumbersome nature of mammalian genetics makes such *in vivo* studies difficult.

Schweichel and Merker's observations of dying cell morphologies suggested that autophagy is induced in and promotes the death of certain types of dying cells [77]. We noted that autophagy and autophagy genes are induced in dying larval salivary gland cells [46, 58], suggesting that this process promotes cell death, but it was not until recently that this has been rigorously tested using genetic approaches. The availability of several *in vivo* models for cell death in *Drosophila* has made it possible to directly test the role of autophagy in cell death. Several *Drosophila* studies demonstrate a positive role for autophagy in cell death. Atg1 over-expression in the *Drosophila* larval fat body is sufficient to induce autophagy and cell death in a caspase-dependent manner [11]. Mutations in *atg8* or *atg18*, or decreased function of *atg1*, in addition to a number of other *atg* genes, all lead to the incomplete destruction of larval salivary glands, structures that die with Type II morphology [73]. Furthermore, knockdown of *atg* genes specifically in salivary gland cells leads to incomplete gland destruction, indicating that the role of autophagy occurring within these dying cells is tissue-autonomous [73]. However, in contrast to the results in the fat body [11], Atg1-over-expression in salivary glands induces cell death in a caspase-independent manner.

The relationship between autophagy and caspases may be context-specific. Several studies have suggested a synergistic role for autophagy and caspases in cell death that occurs during oogenesis in *Drosophila* and higher Dipteran species [86–91]. Two recent studies suggest that there is an epistatic relationship between caspases and autophagy during oogenesis, however their findings differ in important ways (Fig. 1) [92, 93]. Hou et al. recently demonstrated that in germaria and midstage egg chambers, starvation-induced autophagy leads to degeneration [92]. The effector caspase DCP-1 and IAP protein Bruce are required for autophagy induction in these degenerating egg chambers in response to starvation [92]. Therefore this study suggests that caspases function upstream of starvation-induced autophagy and cell death in the ovary [92]. In contrast, Nezis et al. recently demonstrated that during developmentally-induced cell death that occurs during germarium development and mid-oogenesis, both processed caspase3-like expression and autophagy induction occur, however mutations in autophagy genes result in reduced caspase activation [93]. Therefore in this case, developmentally-induced autophagy is required for caspase activation in dying female germline cells [93]. Thus the mechanisms underlying the hierarchical relationship between autophagy and caspases in cells that die during oogenesis remain to be resolved.

Several studies also indicate that caspases and autophagy function in parallel in the destruction of certain tissues during *Drosophila* development (Fig. 1). During the destruction of salivary glands, both autophagy and caspase activation occur, however, expression of the caspase inhibitor p35 only partially prevents salivary gland degradation [46]. In *Drosophila dark* (Apaf-1) mutants, salivary gland degradation is incomplete, but autophagy occurs normally, suggesting a role for this caspase regulator in type II cell death downstream or parallel to autophagy [94]. Significantly, Berry and Baehrecke demonstrate that the combined inhibition of autophagy and caspases enhances the incomplete destruction of salivary glands [73]. A recent study by Mohseni et al. finds that the destruction of the *Drosophila* amnioserosa, an extra-embryonic membrane that is eliminated late in embryogenesis, requires both autophagy and caspase activation [95]. It is not clear in this study whether the relationship between caspases and autophagy is either parallel or epistatic, but this finding provides additional support for the hypothesis that caspases and autophagy function cooperatively in the destruction of tissues during cell death. A similar process may occur in some types of mammalian cells, given that mammalian 3-dimensional cultures of MCF-10A mammary cells die with similar characteristics [96, 97]. Studies of MCF-10A mammary epithelial cell lines, a model for mammary lumen formation, implicate both autophagy and caspases in the elimination of cells during lumen formation [96, 97]. Suppression of either caspase activity or autophagy does not prevent lumen formation, whereas inhibition of both prevents cell elimination [97].

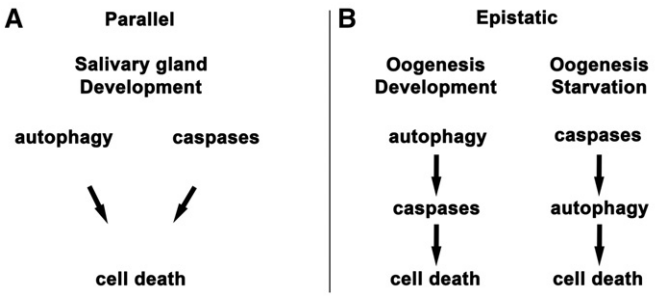

Fig. 1. The relationship between autophagy and caspases during cell death may be context specific. (A) During metamorphosis, *Drosophila* larval salivary glands undergo developmental programmed cell death. During destruction of salivary glands, both autophagy genes and caspases are required for complete degradation, and the combined inhibition of autophagy and caspases enhances this incomplete destruction [73,94]. In addition, caspases are activated in autophagy mutants and autophagy occurs when caspases are inhibited, indicating that autophagy and caspases function in parallel pathways. (B) During *Drosophila* oogenesis, starvation-induced autophagy leads to degeneration of egg chambers, and effector caspase DCP-1 and IAP protein Bruce are required for autophagy induction [92]. During developmentally-induced cell death that occurs during oogenesis, however, mutations in autophagy genes result in reduced caspase activation [93]. These studies indicate that autophagy and caspases function in an epistatic regulatory hierarchy.

Although genetic evidence now indicates that autophagy contributes to cell death in some *in vivo* contexts [73, 92, 93, 95], how autophagy may function in cell death is not completely clear. One possibility is that autophagy is required to generate the metabolic substrates that are required for cell autonomous destruction. It is also possible that autophagy functions to specifically deplete a cell survival factor as has been shown in mammalian cells [98], and with accumulating models in which autophagy functions in physiological cell death, *Drosophila* may provide the best system to discover such a factor. Alternatively, autophagy may deplete critical resources, such as substrates for metabolism and mitochondria, which are required for cell survival, and this may lead to cell death.

5. Autophagy and disease models in *Drosophila*

Drosophila provides an ideal model system for studies of genes that have been implicated in human disease. More than 60% of the genes implicated in human disease have orthologs in flies, and studies in fly disease models have identified novel factors that regulate disease [99, 100]. Whereas knowledge of a gene associated with a human disorder may initially be limited to a genetic locus, the ease of fly genetics, the

ability to generate mutants, knockdown or over-express genes of interest make it possible to identify the function of such genes in flies. In addition, many signaling pathways are highly conserved between flies and humans. Therefore, the use of genetic modifier screens in flies, to look for mutations that enhance or suppress a mutant phenotype, makes it possible to quickly place a gene in the context of a genetic pathway and identify novel factors not previously associated with a disease [99, 100].

Autophagy has been implicated in aging, protein aggregate disorders, neurodegeneration, immunity, and cancer [6–8]. While there are no cancer models in *Drosophila*, fly *atg7* and *atg8* mutants, like *atg* mutant mice, have a decreased life span and exhibit neurodegenerative defects [11, 50, 52, 101, 102]. Autophagy is also required for *Drosophila* innate immune responses [103]. Fly disease models have proven useful in identifying new factors that may regulate disease, and have also provided mechanistic evidence for how autophagy is regulated in the context of neurodegenerative disorders, immune responses, and aging.

6. Protein aggregate disorders

The accumulation of protein aggregates in degenerating neurons is associated with many human neurodegenerative diseases [6]. These protein aggregates are often ubiquitin-positive, suggesting defects in protein degradation by the UPS, and a number of studies have shown that the UPS is impaired in neurodegenerative disorders. However, an increase in the number of autophagosomes in degenerating neurons has also been noted in patients with many polyglutamine diseases, as well as Alzheimer's and Parkinson's. Autophagy gene mutant phenotypes also suggest a role for autophagy in aggregate protein disorders and neurodegeneration. *atg7* conditional knockout mice are impaired in autophagosome formation, and accumulate ubiquitin-positive aggregates [70]. Loss of either *atg5* or *atg7* specifically in neurons leads to the accumulation of ubiquitin-positive aggregates and neurodegeneration in mice [101, 102]. *Drosophila atg7* and *atg8* null mutants also exhibit the accumulation of ubiquitin-positive aggregates in degenerating neurons [50, 51, 104].

Although an increase in the number of autophagosomes is associated with neurodegeneration, whether autophagy functions as a cytoprotective or as a cell death mechanism in this context has been a subject of debate. In both *Drosophila* and mouse models of Huntington disease, TOR inhibition induced autophagy and led to a decrease in huntingtin protein accumulation and cell death, whereas autophagy inhibition enhanced polyglutamine toxicity [105]. In a *Drosophila* model of the neurodegenerative disease spinobulbar muscular atrophy (SBMA), polyglutamine expansion led to cell degeneration and *atg* gene-knockdown enhanced this degeneration [106]. Conversely, induction of autophagy via rapamycin treatment in this study suppressed cell degeneration [106]. Thus, these studies suggest that autophagy plays a cytoprotective role in the context of aggregate protein disorders.

While protein aggregate disorders are characterized by defects in the UPS and autophagy, these two catabolic systems have typically been thought to act independently of one another. However recent studies suggest that there may be a relationship between the UPS and autophagy, and several proteins have been implicated in a possible mechanism linking these processes. In the *Drosophila* model for SBMA mentioned above, genetic impairment of the proteasome led to the induction of autophagy [106]. Furthermore, this study demonstrated that the microtubule-associated protein Histone Deacetylase 6 (HDAC6), previously shown to interact with polyubiquitinated proteins [107], is required for autophagy induction upon proteasome impairment [106]. In addition, expression of HDAC6 during proteasome impairment was sufficient to rescue cell degeneration in an autophagy-dependent manner.

Other proteins have also been implicated in providing a link between autophagy and the UPS. p62/Sequestosome 1 (SQSTM1) binds both poly-ubiquitin and atg8/LC3, and loss of p62 leads to an increase in huntingtin-induced cell death in cell lines [108]. In autophagy-deficient mice, p62 is required for the formation of ubiquitin-positive protein aggregates [109]. Similarly, the *Drosophila* p62 ortholog Ref(2)P is required for ubiquitin-positive aggregate formation in *atg8* mutants [51]. In addition, autophagy-linked FYVE protein (Alfy) localizes to ubiquitinated protein aggregates and autophagosomes, and mutations in its *Drosophila* homolog, *blue cheese*, lead to an accumulation of ubiquitin-positive aggregates in neurons [110, 111]. In line with these findings, *Drosophila* null mutations in *vps15*, the Vps34/Class III PI3K regulatory kinase required for autophagy, exhibit ubiquitin- and Ref(2)P-positive aggregates [112].

Studies in *Drosophila* also suggest a role for endosomal biogenesis genes in aggregate protein disorders. Mutations in *escrt* genes are linked to several human neurodegenerative disorders, including frontotemporal dementia and amyotrophic lateral sclerosis [113, 114]. In a *Drosophila* model for Huntington disease, reducing the genetic dosage of ESCRT proteins, or expressing a dominant-negative form of the ESCRT regulatory ATPase Vps4, led to the accumulation of autophagosomes and ubiquitin-positive aggregates [62, 63]. In ESCRT-depleted HeLa cells, ubiquitin-positive aggregates also contained p62 and Alfy [115]. Rab5, a gene known to be involved in endocytosis, was also shown to be required for autophagosome formation, and inhibition of Rab5 enhanced polyglutamine toxicity in a *Drosophila* model of Huntington disease [116].

7. Autophagy and aging

Drosophila atg7 and *atg8* mutants have a decreased lifespan and are sensitive to oxidative stress, demonstrating a role for autophagy in aging [50, 52]. In *Drosophila*, autophagy gene expression is reduced with age [52]. Conversely, Atg8 over-expression in adult neurons results in an increase in resistance to oxidative stress, a process that has been linked to aging [117], and the extension of lifespan [52]. Further, *atg7* conditional knockout mice accumulate peroxisomes in their livers and exhibit oxidative stress, demonstrating a direct role for autophagy in peroxisomal degradation in mammals [118].

Evidence in mice suggests that the relationship between autophagy and aging involves chaperone-mediated autophagy (CMA) [119]. CMA is a selective degradation process in which soluble proteins are delivered directly to the lysosome [2]. Like macroautophagy, CMA is activated in response to oxidative stress [120, 121]. In addition, LAMP2A, a receptor required for CMA, decreases with age due to a loss in LAMP2A protein stability [122, 123]. Furthermore, inducing the expression of LAMP2A in the livers of transgenic mice increases the levels of CMA, and these mice accumulate fewer damaged proteins in their livers [119]. CMA has not been investigated in *Drosophila*, and it would be useful to apply the strength of fly genetics to this important catabolic process.

8. Autophagy and immunity

Evidence suggests that autophagy plays important roles in mammalian innate and adaptive immunity in the elimination of pathogens, and antigen processing and presentation [8, 124]. Autophagy is important during T-cell selection to generate self-tolerance, and autophagy deficiency leads to multi-organ inflammation [125]. Furthermore, mutations in *atg16* have been linked to inflammatory bowel disease in humans and mice [126, 127]. Autophagy is also important as a host-defense mechanism to clear intracellular pathogens [128–132], and some invading pathogens and viruses utilize or inhibit the autophagic machinery to evade host-defense mechanisms [133–135]. During *M. tuberculosis* infection, autophagy is induced, and this induction requires the immunity-related GTPase family member IRGM [129, 136]. However, mechanistic

evidence demonstrating how autophagy is regulated in the context of immune signaling has been lacking.

Drosophila lack adaptive immunity, but can mount innate immune responses. Recent *in vivo* studies in *Drosophila* have helped to elucidate the mechanism of autophagy induction during pathogenic infection. Yano et al. found that *Drosophila* resistance to the gram-positive bacteria *L. monocytogenes* requires the pattern-recognition receptor PGRP-LE, a member of a family of receptors that recognize bacterial peptidoglycans, induce antimicrobial peptide (AMP) genes, and activate the innate immune signaling IMD and Toll pathways [137]. Significantly, Yano et al. showed that autophagy in blood cells is necessary to inhibit the intracellular growth of *L. monocytogenes*, and to survive infection. Further, the PGRP-LE receptor is required for the induction of autophagy following *L. monocytogenes* infection [137]. However, autophagy induction in this case is independent of the Toll and IMD immune signaling pathways, suggesting the possibility that the PGRP-LE receptor may signal through an unknown factor to induce autophagy [137].

Another recent *Drosophila* study has provided new information about the crosstalk between autophagy and innate immune signaling pathways. A screen for genes required for *Drosophila* innate immune responses to bacterial infection identified the gene immune response-deficient 1 (*ird1*), the *Drosophila* homolog of the Vps34/Class III PI3K regulator, *vps15* [138]. AMP genes are induced during starvation [139], but *ird1* mutants fail to express AMP genes upon infection [138]. Furthermore, *ird1* mutants cannot activate the IMD pathway, demonstrating that *ird1*/*vps15* is an important regulator of the innate immune response [138]. In addition, this study showed that if wild-type flies are starved during bacterial infection, the expression of AMP genes is suppressed, and these flies exhibit decreased survival upon infection, suggesting that autophagy plays an important role in modulating nutrient sensing and immune signaling [138]. This result provides insight into studies suggesting that in human and mouse populations, malnutrition leads to an increased susceptibility to infections [140–142].

9. Conclusions

Drosophila is well-suited to *in vivo* studies of autophagy and its relationship to cell survival, growth, and death in the context of a developing multi-cellular organism. *Drosophila* research has produced many new discoveries about the role and regulation of autophagy during development, aging, innate immunity, and in the context of diseases such as protein aggregate disorders and neurodegeneration. Genetic screens for genes that are required for autophagy in *Drosophila* have yielded new discoveries [143, 144], but additional screens are needed to identify the genes that regulate autophagy in the context of nutrient deprivation, pathogenic invasion, and other processes. Importantly, these screens should provide key mechanistic information about the regulation of autophagy, and it is likely that such genes will have conserved functions in mammals.

Autophagy is induced in *Drosophila* upon starvation in tissues such as the fat body, muscle, and ovaries. Autophagy is also induced in response to the steroid hormone ecdysone during metamorphosis in tissues such as the fat body, intestine, and salivary glands. This allows studies to be carried out in different cell types and in response to different stimuli. Autophagy regulation may differ in the contexts of nutrient deprivation compared to ecdysone induction, in the context of cell survival versus cell death, or in response to different pathogens. Thus, there may be a cell-context-specific component to the proper regulation of autophagy, and it is possible that as-yet undiscovered regulators that influence the induction of autophagy in response to different stimuli, and/or in specific cell types, may exist, and flies are well-suited to such discoveries.

Acknowledgement

We thank the NIH (GM59136 and GM079431) for support.

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