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Review

Autophagic programmed cell death in *Drosophila*

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Abstract

Autophagic programmed cell death occurs during the development of diverse animal groups, but the mechanisms that control this genetically regulated form of cell killing are poorly understood. Genetic studies of bulk protein degradation in yeast have provided important advances in our understanding of autophagy, and recent investigations of *Drosophila* autophagic cell death suggest that some of these mechanisms may be conserved. In *Drosophila*, several steroid-regulated genes that encode transcription regulators are required for autophagic cell death. These transcription regulators appear to activate a large number of genes that play a more direct role in cell killing, including genes that function in apoptosis such as caspases. While caspase function is required for autophagic cell death during *Drosophila* development, genes encoding proteins that are similar to the yeast autophagy regulators are also induced in dying salivary glands. Furthermore, numerous noncaspase proteases, cytoplasmic organizing factors, signaling molecules, and unknown factors are expressed in interesting patterns during autophagic cell death. This article reviews the current knowledge of the regulation of autophagic programmed cell death during development of *Drosophila*.
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Keywords: autophagy; apoptosis; proteolysis; cytoplasmic degradation; *Drosophila*

Abbreviations: dark, fly Apaf-1 homolog ark; Nc, the caspase dronc

Introduction

Schweichel and Merker[1] recognized that at least three forms of cell death occur during development of animals based on the location and role of lysosomes. While nonlysosomal cell death has not been commonly observed, apoptosis and autophagic programmed cell death are prominent in developing animals of diverse taxa.[2] Apoptotic cells exhibit condensation of the nucleus and cytoplasm, fragmentation, and they are engulfed by phagocytes where the lysosome completes degradation of the dying cell.[3] Unlike apoptosis, cells that die by autophagic cell death are degraded with little or no help from phagocytes and utilize lysosomes within the dying cell for degradation. Defects in apoptosis have been associated with a variety of human disorders including cancer,[4] and studies of the mechanisms underlying apoptosis have been the focus of cell death research.[5,6] In contrast, little is known about the mechanisms that regulate autophagic cell death.

The degradation of cytoplasmic organelles and proteins is an important component of proper cell life and death. While the ubiquitin/proteosome pathway is used to degrade many short-lived regulatory proteins,[7] long-lived proteins are targeted to the lysosome by five mechanisms including direct transport, vacuolar import and degradation, endocytosis, microautophagy, and macroautophagy.[8] Macroautophagy (hereafter refered to as autophagy) is the best known mechanism for sequestration of long-lived proteins and cytoplasmic components into the lysosomal compartment of the cell for degradation. The first descriptions of lysosomal structures that contained cytoplasmic components such as mitochondria were observed in rat livers perfused with glucagon[9] and rat hepatomas.[10] These autophagic structures have since been described in organisms that are as different as yeast and humans. During autophagy, cytoplasm and organelles are sequestered in double-membrane vesicles known as autophagic vacuoles in organisms such as insects and mammals and as autophagosomes in yeast[11] (Figure 1). Although the origin of autophagic vacuole membranes are a subject of debate, evidence indicates that they are derived from the rough endoplasmic reticulum.[12] Autophagic vacuoles then dock against the lysosomal vacuole, and fusion of the outer autophagic vacuole membrane with the lysosomal membrane releases the inner membrane-bound cytoplasmic contents into the lysosome where degradation occurs.

Autophagic structures were first associated with developmental programmed cell death in insects[13,14] and chickens,[15] and it is now clear that autophagic cell death occurs during development of diverse organisms including humans.[2,16–18] The recent association of autophagic cell death with models of tumorigenesis[19–22] and neurodegenerative disorders[23–26] has led to greater interest in the mechanisms that regulate this form of cell destruction.

Lessons from yeast

The molecular genetic basis of protein and organelle turnover by autophagy has been well characterized in the yeast *Saccharomyces cerevisiae*. In yeast, autophagy is induced under nutrient-limiting conditions. Genetic screens for yeast that either lacked accumulation of autophagic structures or exhibited defects in the degradation of cytoplasmic proteins under starvation conditions resulted in the identification of the *apg*[27] and *aut*[28] mutants. In addition, the isolation of altered

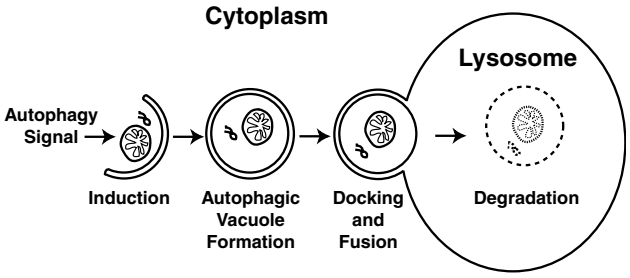

Figure 1 Diagram of the steps in autophagy. Autophagy is induced by starvation in yeast and cell death signals such as steroids during autophagic cell death in *Drosophila*. Induction results in the formation of a double-membraned autophagic vacuole that encloses cytoplasmic components including organelles such as mitochondria. The autophagic vacuole then docks against the lysosome, and the fusion of the outer autophagic vacuole membrane with the lysosome releases the inner membrane-bound cytoplasmic components into the lysosome where degradation occurs. Redrawn after Klionsky and Emr[11]

cytoplasm to vacuole-targeting mutants (*cvt*) in screens of yeast for defects in proteolytic processing of aminopeptidase I[29] led to the recognition of the genetic overlap between the *apg*, *aut*, and *cvt* pathways.[30] In total, 19 *apg*, *aut*, and *cvt* genes have been identified that regulate the induction, formation, size, and breakdown steps in yeast autophagy (reviewed in Ohsumi[8] and Kilonsky and Emr[11]).

Genetic and biochemical studies of yeast indicate that the *apg*, *aut*, and *cvt* genes have distinct and related functions. While the role of some of these genes remains mysterious, two related ubiquitin-like systems are required for yeast autophagy. APG12 and AUT7/APG8/CVT5 encode proteins that show no apparent homology to ubiquitin, but both of these proteins appear to function in ubiquitin-like conjugation systems.[31,32] APG12 and AUT7/APG8/CVT5 are both activated by the E1-like protein APG7/CVT2.[33,34] APG12 is then conjugated to the E2-like protein APG10,[35] while AUT7/APG8/CVT5 becomes conjugated to the E2-like protein AUT1/APG3.[31] APG12 is then conjugated to APG5[32], and AUT7/APG8/CVT5 is attached to the lipid phosphatidylethanolamine.[31] Mutations in either of these pathways prevent the proper formation of autophagic vacuoles,[27] and indicate that ubiquitin-like systems are involved in the bulk degradation of long-lived cytoplasmic proteins. While it is not clear if the genes that are required for yeast autophagy are also required for the formation of autophagic structures during programmed cell death, recent studies in *Drosophila* suggest similarity in the mechanisms of cytoplasmic degradation.[36]

Activation of autophagic cell death in *Drosophila*

The steroid hormone 20-hydroxyecdysone (ecdysone) titer begins to rise 10 h after puparium formation, and reaches its peak level 12 h after puparium formation when it triggers the synchronous death of larval salivary gland cells.[37,38] This cell death is preceded by markers of apoptosis including DNA fragmentation and nuclear acridine orange staining.[39] While these data suggest that salivary glands die by apoptosis, their morphology indicates that they die by autophagy (Figure 2). At 1 h after this peak in ecdysone titer (13 h after puparium formation), salivary gland cells become round in shape, large cytoplasmic vacuoles appear to fragment, and a second class of vacuoles appears that is associated with the plasma membrane.[40] By 14 h after puparium formation, salivary glands possess autophagic vacuoles that contain cytoplasmic structures including mitochondria.[40] The cellular changes that occur following the rise in ecdysone titer are accompanied by changes in the tubulin and actin cytoskeleton, and accumulation of acid phosphatase activity.[41] Salivary glands are destroyed 16-h following puparium formation. These changes in cell morphology indicate that the production and movement of vacuoles, reorganization of the cytoplasm and cell shape, and cytoplasmic degradation by proteases may all be involved in the autophagic destruction of salivary glands.

Drosophila salivary glands have served as a long-standing model for studies of steroid signaling.[42–44] Ecdysone activates salivary gland cell death through a transcriptional regulatory hierarchy (Figure 3). The ecdysone receptor is encoded by the nuclear receptor genes *EcR* and *usp*.[45–50] The ecdysone receptor complex, and the nuclear receptor competence factor *βFTZ-F1*, activate transcription of the early genes *BR-C*, *E74A*, and *E93*.[51,52] Salivary glands are not destroyed in animals with mutations in *βFTZ-F1*, *BR-C*, *E74A*, and *E93*.[52-55] While *βFTZ-F1* and *E93* mutants exhibit defects in vacuolar changes during salivary gland autophagic cell death, *BR-C* and *E74A* mutant salivary glands progress to a very late stage in cell death even though complete destruction is prevented.[40] In addition, *E93* mutants exhibit similar defects in the formation of autophagic vacuoles in dying midgut cells,[56] indicating that *E93* is required for autophagy in dying *Drosophila* cells. *BR-C*, *E74A*, and *E93* all encode proteins that regulate transcription of target late genes,[54,55,57,58] and these downstream genes appear to function more directly in programmed cell death.

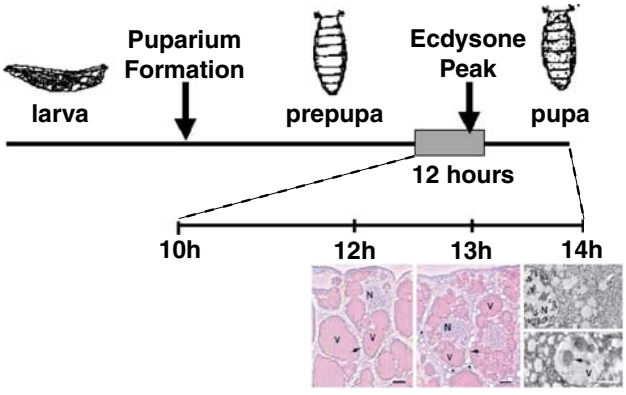

Figure 2 Steroid regulation of autophagic programmed cell death in *Drosophila* salivary glands. At 10 h after puparium formation, the ecdysone titer starts to rise peaking 12 h after puparium formation. This pulse of steroid triggers the autophagic destruction of salivary glands, and this process is complete 16 h after puparium formation. Following the peak in ecdyone, cells become round in shape, large eosin-positive vacuoles (V) appear to fragment, and a new class of eosin-negative vacuoles (*) appear along the plasma membrane, and the nuclei (N) remains intact even though DNA fragmentation has taken place. At 14 hours after puparium formation, autophagic vacuoles containing mitochondria are present in the cytoplasm of salivary gland cells (arrows). Data reproduced from Lee and Baehrecke[40]

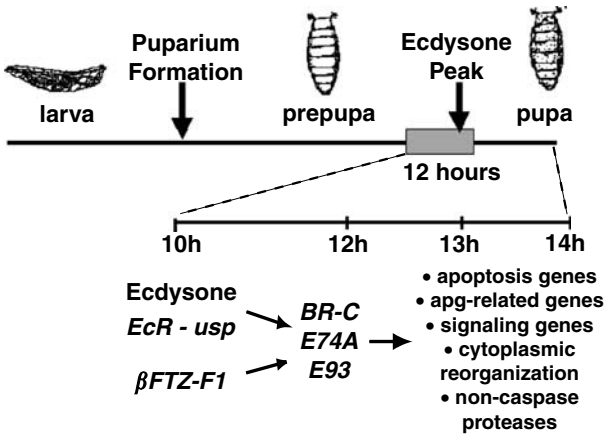

Figure 3 Model for genetic regulation of autophagic programmed cell death in *Drosophila* salivary glands. At 10 h after puparium formation, the ecdysone titer rises and this steroid is bound by the receptor complex that is encoded by *EcR* and *usp*. The ecdysone receptor complex and *βFTZ-F1* activate the *BR-C*, *E74A*, and *E93* early genes. *BR-C*, *E74A*, and *E93* regulate genes involved in signaling, cytoplasmic reorganization, proteolysis, autophagy (*apg*-related genes), and apoptosis

Apoptosis genes function in *Drosophila* autophagic cell death

Several lines of evidence indicate that the core apoptosis machinery functions in autophagic cell death during *Drosophila* development. Autophagic cell death of *Drosophila* salivary glands and midguts is foreshadowed by markers of apoptosis including DNA fragmentation.[39,40,56] In addition, destruction of salivary glands and midguts is prevented by expression of the caspase inhibitor p35.[39,40,56] Significantly, the proapoptotic genes *rpr* and *hid* (*W*) increase in transcription following the rise in ecdysone that triggers salivary autophagic cell death, and this change is concurrent with a decrease in the inhibitor of apoptosis *diap2*.[39] At the same time, the fly *Apaf-1* homologue *ark* (*dark*, *dapaf1*, *hac1*), the caspases *dronc* (*Nc*), *drice* (*Ice*), and *dream* (*strica*), the Bcl-2 family member *buffy* (*dborg-2*), the CD36 realtive *crq*, and the DNase *rep4,* all exhibit dramatic increases in transcription just before salivary gland autophagic cell death.[36,59,60]

Animals with mutations in the transcription regulators *BR-C*, *E74A*, and *E93* prevent proper transcription of apoptosis genes (Figure 3). Both *BR-C* and *E93* mutant salivary glands have dramatically reduced levels of *rpr*, *hid*, *dronc*, *dream*, and *crq*.[36,59] While *buffy* and *drice* RNA levels are also decreased in *E93* mutant salivary glands, *drice* is not altered and *buffy* is ectopically expressed in *BR-C* mutants.[36] *E74A* mutants exhibit reduced levels of *hid*, *crq*, *buffy*, *drice*, and *dream*.[36,59] Studies of apoptosis gene promoters indicate that *rpr* is a direct target of the ecdysone receptor[54] and that *dronc* is a direct target of BR-C.[61] These data suggest that the ecdysone-regulated early genes *BR-C*, *E74A*, and *E93* have overlapping and distinct target apoptosis genes that they regulate. Significantly, it appears that proper transciption of apoptosis genes is essential for autophagic cell death, since animals with mutations in *BR-C*, *E74A*, and *E93* prevent destruction of salivary glands. This is intriguing, as studies of the human mammary carcinoma cell line MCF-7 indicate that caspases are not required for autophagic death of these cells.[62]

Yeast autophagy genes appear to be conserved in higher animals, and are induced during autophagic programmed cell death

In all, 11 of the 19 genes that are required for yeast autophagy encode related genes in flies and humans (hereafter refered to as *apg*-related genes)[16,63] (Table 1). Nine of these 11 *apg*-related genes are transcribed in dying *Drosophila* glands, and the seven genes that are most similar to *apg2* (*CG1241*), *apg3* (*CG6877*), *apg4* (*CG6194*), *apg5* (*CG1643*), *apg7* (*CG5489*), *apg9* (*CG3615*), and *aut10/cvt18* (*CG7986*) exhibit increased transcription following the rise in steroid hormone that triggers autophagic programmed cell death of this tissue.[36,64] It is interesting that flies possess genes that are related to the yeast genes involved in both of the autophagy ubiquitin conjugation systems, and that they are also the genes that increase in transcription following the rise in steroid that triggers autophagic cell death. *CG1241* encodes a novel protein that is similar to APG2 that functions in the formation and completion of autophagic vacuoles.[65] APG2 interacts with the integral membrane protein APG9/AUT9/CVT7,[66] which is similar to the predicted protein encoded by *CG3615*. *CG7986* encodes a predicted protein that is most similar to AUT10/CVT18 which has WD40 domains, functions in the formation of autophagic vacuoles, and is required for proper localization of APG2.[67] *CG6194* encodes a predicted protein that is similar to the AUT2/APG4 cysteine protease, which interacts with and processes AUT7/APG8/CVT5, and is involved in the formation and completion of autophagic vacuoles.[68] AUT7/APG8/CVT5 is conjugated with the ubiquitin-E1-like enzyme APG7/CVT2,[31] which is similar to the protein encoded by *CG5489*, indicating that one of the ubiquitin-like conjugation systems may function in salivary gland autophagy. AUT7/APG8/CVT5 is also conjugated with the ubiquitin-E2-like enzyme AUT1/APG3.[31] AUT1/APG3 is similar to the predicted protein encoded by *CG6877*, suggesting the possibility that the second ubiquitin-like conjugation system functions in salivary gland autophagy. *CG1643* encodes a predicted protein that is similar to the novel protein APG5,[69] which also functions in the formation of

Table 1 Yeast autophagy proteins, their biochemical properties, and the *Drosophila* gene encoding the most similar predicted protein

Yeast protein	Biochemical properties	*Drosophila* gene
APG1/AUT3/CVT10	Serine/threonine kinase	*CG8866*
APG2/AUT8	Interacts with APG9	*CG1241*
AUT1/APG3	E2-like, conjugated with AUT7/APG8/CVT5	*CG6877*
AUT2/APG4	Cysteine protease, interacts with AUT7/APG8/CVT5	*CG6194*
APG5	Conjugated with APG12	*CG1643*
APG6	Interacts with APG14	*CG5429*
APG7/CVT2	E1-like, conjugated with APG12 or AUT7/APG8/CVT5	*CG5489*
AUT7/APG8/CVT5	Conjugated with APG7/CVT2, AUT1/APG3	*CG32672*
APG9/AUT9/CVT7	Integral membrane protein, interacts with APG2	*CG3615*
APG12	Conjugated with APG7/CVT2, APG10, APG5	*CG10861*
AUT10/CVT18	WD40 domains	*CG7986*

Drosophila melanogaster genes were identified by using BLAST searches of the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov./). BLAST scores lower than 10^{-5} were considered similar. Yeast genes that function in autophagy, but exhibit low similarity to predicted fly genes, are not included

autophagic vacuoles. APG5 is conjugated to APG12, and this complex is also activated by the ubiquitin-E1-like enzyme APG7/CVT2,[32] further indicating that both ubiquitin-like conjugation systems that are used during yeast autophagy may also function in salivary gland cell death.

Mutations in the ecdysone-regulated transcription factor genes that prevent proper salivary gland cell death also inhibit proper transcription of the *apg*-related genes.[36] Animals with mutations in *BR-C* exhibit ectopic transcription of *CG6194* (*apg4*-like) and *CG1643* (*apg5*-like), and decreased transcription of *CG5489* (*apg7*-like) in salivary glands at the stage that they would normally die. While animals with mutations in *E74A* only exhibit decreased transcription of *CG6194*, salivary glands dissected from *E93* mutants have decreased levels of *CG1241* (*apg2*-like), *CG6194*, *CG1643*, and *CG5489*. Yeasts with mutations in *apg4*, *apg5*, and *apg7* are defective in autophagic vacuole formation.[27] Thus, it is striking that *E93* mutants exhibit decreased *CG6194*, *CG1643*, and *CG5489* RNA levels, since *E93* mutant salivary gland and midgut cells are defective in the formation of autophagic vacuoles.[40,56]

Additional factors that are induced during *Drosophila* autophagic cell death

Genomewide studies of dying cells have identified numerous interesting genes that are induced just prior to autophagic programmed cell death (Figure 3). For example, several signaling molecules and transcription regulators increase in transcription in dying salivary glands. The *Drosophila* gene *CG8304* encodes a serine/threonine kinase that is most similar to the death-associated protein kinase (DAPk) in humans, and transcription of this gene increases 36-fold in dying salivary glands.[36] DAPk mediates membrane blebbing and formation of vesicles in dying human cell lines,[70] and suggests that *CG8304* may have a related function in *Drosophila*. Steroids usually function by activating transcription and, therefore, it is not surprising that transcription regulators are induced in dying salivary glands including the corepressors (*Smarter* and *CG4756*), the NFkB regulator *cactus*, the NFkB family member *dif*, several other genes encoding DNA-binding proteins (*bun*, *sox14*, *CG8319*), and components of transcription complexes (*trap95* and *hsf*).[36,64]

Salivary gland cells exhibit dynamic changes in cell shape and vacuole localization just before autophagic cell destruction, suggesting that cytoplasmic reorganization and proteolysis are important for their death. Consistent with this notion, genes encoding motor proteins including *ctp*, *ck*, and *dlc90F*, and members of the Rho, Rac, and Rab families of small guanosine triphosphates (GTPases) exhibited increase in transcription just before salivary gland autophagic cell death.[36,64] While cell remodeling is known to play an important role in phagocyte engulfment of apoptotic cells,[71] the role of these factors in autophagic cell death is less clear. In addition to caspases, several cysteine, serine, and metalloproteases increase in transcription in dying salivary glands, and these changes in RNA level are complemented by decreased transcription of cysteine, serine, and metalloprotease inhibitors.[36,64] Furthermore, upregulation of the matrix metalloprotease *mmp1* is abolished in mutants that prevent salivary gland cell death.[36] These proteases likely complement caspase function during autophagic cell death, as mutations in *mmp2* prevent proper destruction of *Drosophila* midgets,[72] this tissue dies by autophagic cell death,[56] and inhibition of caspases does not completely prevent autophagic changes in dying cells.[40]

The most important advances in science often come from the exploration of the unknown. Therefore, it is interesting that many genes encoding novel factors are induced in dying cells. We have identified genes that increase following both radiation-induced apoptosis and steroid-triggered autophagy. Very few common genes were identified using this approach, but the genes *CG10965*, *CG17323*, *CG7144*, *EG25E8.4*, and *CG5254* increased in RNA levels during both ecdysone-and radiation-triggered cell death.[36] Transcription of these genes was also altered in salivary glands of *BR-C*, *E74A*, and *E93* mutants, suggesting a possible functional role for these genes in autophagic programmed cell death.

Concluding Remarks and Future Directions

Studies of developing animals indicate that at least two forms of programmed cell death have been conserved during evolution.[2] Studies of apoptosis in cell lines indicate that the

biochemical mechanisms underlying programmed cell death are similar in different cell types. Dying cell morphology can be very different,[73] however, and identifying the mechanisms that regulate different types of physiological cell death will lead to a better understanding of how defects in this fundamental cellular process lead to aberrations in animal growth and development.

The steroid ecdysone triggers autophagic cell death during *Drosophila* development, but other factors must provide competence signals for cells to be capable of dying. These 'competence factors' may include cell age, status in the cell cycle, the presence of a transcriptional activator, absence of a transcriptional repressor, or the state of chromatin to name a few possibilities. Ultimately, this steroid signal activates a group of transcription regulators that include *BR-C*, *E74A*, and *E93*. These early genes appear to regulate a complex series of downstream cellular events that include cytoplasmic reorganization including cell shape and vacuole transport, proteolysis by caspase and apparently noncaspase proteases, genes related to yeast genes that function in autophagy, and many unknowns.

Many gaps exist in our knowledge of the mechanism underlying autophagic cell death, and this is an exciting time to ask critical questions about this understudied form of cell destruction. How many of the genes that are expressed in dying cells are required, and is it critical for these factors to integrate in a specific temporal manner? Do the *apg*-related genes serve the same purpose in yeast and higher animals? Are the noncaspase genes that are used during autophagy used during apoptosis? What is the clinical relevance of autophagic programmed cell death? Given our limited knowledge of the mechanisms underlying autophagic programmed cell death, it is clear that this fundamental cellular process will be a fruitful area of investigation for years to come.

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