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Distinct death mechanisms in *Drosophila* development

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Abstract

Apoptosis and autophagic cell death occur during *Drosophila* development, and recent advances in their mechanisms have been made. As in other organisms, apoptosis is executed by caspases. In living cells, caspases are kept in check through a combination of IAP-binding and proteolytic inhibition. Once a cell commits to apoptosis, phagocytes recognize them through the immuno-receptor-like proteins Draper and Simu, and initiate corpse engulfment. *Drosophila* research has significantly contributed to the idea that autophagy is required for certain forms of cell death, and that caspase function in autophagic cell death depends on cell context. Surprisingly, the cell corpse engulfment receptor Draper also functions in autophagic cell death. These advances facilitate our understanding of the cell death mechanisms in development and disease.

Introduction

Drosophila melanogaster serves as a popular model for cell death research, in part, due to powerful genetic tools that have been developed throughout the past century. In particular, much progress has been made in understanding *Drosophila* “apoptosis”, a term used to describe dying cells that undergo condensation of cell volume and phagocytosis [1,2] (figure 1). More recently, *Drosophila* has contributed significantly to establishing the idea of autophagic cell death, a process that involves self-devouring of cellular content [3]. On the other hand, necrosis, as defined by swelling and rupture of dying cells, remains poorly characterized in this organism. There are cases of Programmed Cell Death (PCD) that do not fit into the existing definitions of apoptosis/necrosis/autophagic cell death. For example, adult wing cells activate a communal cell death program soon after eclosion with the help of well-established pro-apoptotic genes, but the dying cells are not engulfed by phagocytosis [4]. In this review, we will focus on genes and mechanisms that underlie apoptosis and autophagic cell death. While these cell death regulators also engage in non-apoptotic processes, like cell differentiation and proliferation, those topics will not be covered here as they have been extensively reviewed elsewhere [5].

The initiators of Apoptosis in *Drosophila*, reaper, hid and grim

There are numerous causes of apoptosis, including those caused by stress or even cell-fate determining transcription factors. In *Drosophila*, these various causes converge on the

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induction of three pro-apoptotic genes, called *grim*, *reaper* and *hid*, which serve as the initiating triggers of the apoptosis program (reviewed in [6]). When these three genes are lacking, *Drosophila* embryos become virtually devoid of apoptosis. Grim, Reaper and Hid proteins share a small motif conserved in their N-terminus, widely known as IBM (IAP-binding motif). Functionally, the three proteins act by inhibiting the IAP family of proteins, and are thus referred to as IAP-antagonists (figure 1). Recent evidence indicates that IAP-antagonists must bind each other and form higher order molecular complexes in order to efficiently trigger apoptosis[7].

As *Drosophila* apoptosis requires the expression of IAP-antagonists, the regulatory sequences in their locus must somehow “interpret” the many pro-apoptotic stress and signaling pathways. Reflecting the large number of such apoptotic stimuli, this locus spans more than 150 kb, with a striking level of sequence conservation with other *Drosophila* species [8]. In addition to containing specific binding sites for mediators of apoptosis [9] [10], this locus is regulated epigenetically: The chromatin structure adopts a heterochromatin-like state during mid-embryogenesis, disabling cells from inducing IAP-antagonist expression in response to injury[8].

Inhibitors of Apoptosis

Grim, Reaper and Hid initiate apoptosis primary through antagonizing Inhibitor of Apoptosis Proteins (IAP). There are at least three members of the IAP family in *Drosophila*, which all contain signature Baculovirus IAP Repeat (BIR) domains. Diap2 can inhibit a specific effector caspase called drICE [11], but its overall effect on apoptosis appears minor compared to its cousins. Another member of the *Drosophila* IAP family, *bruce*, encodes a ubiquitin-conjugating enzyme that suppresses *grim* and *reaper*-induced apoptosis [12,13]. Diap1 has an overall sequence that is similar to Diap2, but plays a highly visible role in apoptosis: Loss of Diap1 protein leads to an immediate activation of apoptosis in virtually all somatic cells analyzed, while overexpression of this gene strongly inhibits *grim*, *reaper* and *hid*'s pro-apoptotic activity. The mechanistic basis of Diap1 function have been extensively reviewed elsewhere [14]. In brief, Diap1 exerts a strong influence on apoptosis, in part, by physically interacting with a number of key pro-apoptotic proteins, including caspases that execute apoptosis. Once bound to its targets, Diap1 adds poly-ubiquitin chains to substrates. For most targets, the poly-ubiquitin chains are recognized by the proteasome for degradation. Under certain conditions, however, Diap1 can add poly-ubiquitin chains with an alternative configuration, with ubiquitins linked through the Lys63 residue. In this configuration, the substrate protein function is often inhibited without degradation [15]. An example of such a Diap1 substrate includes the effector caspase drICE [16]. While Diap1 primarily ubiquitinates pro-apoptotic proteins in living cells, it is itself ubiquitylated for degradation under apoptotic conditions, allowing unbridled caspase activation (reviewed in [14]). Among those that stimulate IAP auto-ubiquitylation are IAP-antagonists. It has been suggested that IAP-antagonists help Diap1 translocate to the mitochondrial membrane, where additional factors required for Diap1 auto-ubiquitylation reside [17].

Caspase activation

In mammals, apoptosis is prominently regulated by Bcl-2 family proteins, which control the release of cytochrome c into the cytoplasm to promote the formation of the apoptosome, a platform for caspase activation (reviewed in [18]). In *Drosophila*, however, the significance of this “mitochondrial” pathway remains questionable. For example, loss-of-function condition for a *Drosophila* Bcl-2 family member, *Debcl*, does not affect caspase-mediated apoptosis [19]. Moreover, the crystal structure of the *Drosophila* apoptosome holoenzyme reveals oligomers of just two protein components, Apaf-1 related killer (Ark) and the

initiator caspase Dronc, but without cytochrome c [20]. While these studies suggested that living *Drosophila* cells may have constitutive apoptosomes activation, recent studies show that this dangerous death machinery is more rigorously controlled through feedback inhibition among the apoptosome components. Specifically, active Dronc cleaves and degrades Apaf-1 related killer, thereby eliminating the pressure to activate more caspases [21]. Conversely, excessive Apaf-1 related killer somehow stimulates Dronc degradation in a Diap1-dependent way [22]. The net effect is that apoptosome holoenzymes cannot assemble to significant levels in living cells. While it is unclear whether mammals have similar mechanisms of apoptosome regulation, certain cancer cells are known to have low Apaf-1 due to caspase-mediated proteolysis [23].

A recent genome-wide RNAi screen revealed a role of an unexpected gene, TANGO7, which was originally identified as a regulator of ER to Golgi protein trafficking [24,25]. The discovery shows that there is much to learn about caspase activation in flies as well as in humans.

Survival signaling and apoptosis

Apoptosis regulation through survival factors has drawn particular interest due to its implication in tumor formation. Spitz/Ras/MAP Kinase signaling is one such pathway leading to hid phosphorylation and inhibition [26,27]. Other survival signaling pathways regulate Diap1. For example, loss of vps25 in flies causes tumor growth, in part, by inducing Jak/Stat and Hippo signaling pathways, ultimately leading to higher levels of Diap1 expression [28]. The Diap1 promoter has functional binding sites for Stat and Scalloped (Sd), which are transcription factors mediating these pathways [29-31].

Engulfment of cell corpse

One of the original definitions of “apoptosis” includes the engulfment of cell corpse. In *Drosophila* embryos, apoptotic cells are engulfed by circulating macrophages [32], while in larval imaginal discs, epithelial cells can engulf neighboring apoptotic bodies [33,34]. In the nervous system, glial cells act as phagocytes [35,36]. A series of recent studies have shown that the apoptotic corpse engulfment machinery also help eliminate necrotic corpses, fragmented axons and synaptic boutons that fail to form stable postsynaptic contacts [37,38]. In addition, recent evidence shows that phagocytosis is not a process that merely follows cell death, but rather, affects the onset of apoptosis [34]. Together, these observations indicate that the cell corpse engulfment machinery has much broader roles than had been previously thought.

In molecular terms, phagocytosis is thought to begin with an “eat me” signal displayed on the apoptotic cell body. Two types of phagocytic receptors are implicated in the recognition of these signals: CD36-like tethering signals without a significant intracellular domain, such as Croquemort plays a role primarily in embryonic cell corpse engulfment by macrophages [32]. While the effectors of Croquemort remain poorly understood, mutations in pallbearer, encoding an F-box protein, reportedly shows phagocytosis defects similar to Croquemort [39]. In addition, docking receptors with noncatalytic intracellular domains are required for cell corpse engulfment. These include *Drosophila* Draper and Simu [35,40], which appear to have non-redundant functions. Simu is required for the glial cells to make contact with the cell corpse [35]. Draper also binds directly to a ligand, pretaporter, which is exposed on the surface of apoptotic cells [41]. Once activated, Draper becomes phosphorylated in its cytoplasmic domain, which serves as a docking site for a non-receptor kinase, Shark [42]. Signaling downstream of Draper appears to involve genes that regulate Ca²⁺ homeostasis: These include *undertaker* and ryanodine receptors [43]. In an independent study, mutations in *trpml*, a member of the TRP family of [44] cation conducting channels, was found to be

defective in cell corpse engulfment. Interestingly, mutations in the human TRPML1 gene underlie an early childhood neurodegenerative disorder known as mucopolipidosis IV[45-47], thereby implicating cell corpse clearance defect in human disease.

Autophagic cell death

Autophagy (self-eating) involves the delivery of cytoplasmic material to the lysosome (reviewed in [48]). During macro-autophagy (hereafter referred to as autophagy), double membrane vesicles called autophagosomes form around cytoplasmic cargo, including proteins and organelles, and the fusion of the outer autophagosome membrane with the lysosome results in degradation of the cargo in the autolysosome. Studies of starving yeast led to the identification of the *Atg* genes that are required for autophagy[49-51]. These genes are conserved regulators of autophagy in organisms that are as diverse as yeast, flies and humans (reviewed in [48]).

Autophagic cell death was morphologically defined by the presence of autophagosomes and autolysosomes in the dying cell[52]. Therefore, these dying cells were thought to use autophagy and the lysosome in the dying cell for degradation, while having little to no association with phagocytes [53,54]. The role of autophagy in cell death has been controversial because of the clear association of autophagy with cell survival[55,56]. While reports of autophagy functioning in the death of derived cultured mammalian cells have contributed to significantly to advancing interest in this alternative form of cell death[57,58], studies of autophagic cell death during development of *Drosophila* have significantly advanced our understanding of the importance of autophagy in dying cells under physiological conditions.

Although autophagic cell death has been described in a wide variety of organisms[53], mechanistic studies of the function of *Atg* genes in cell death under physiological conditions were first conducted in *Drosophila*. *Drosophila* larval salivary glands possess the morphology of cells that die by autophagic cell death[59], and *Atg* genes are induced just hours before cell death[60,61]. Significantly, *Atg* genes are required for the clearance of salivary gland cells[62]. In addition, expression of the *Atg1* kinase in either salivary glands or the fatbody is sufficient to induce autophagy and kill these cells[62,63]. This *Atg1*-induced cell death requires the function of other down-stream *Atg* genes[62,63], indicating that this cell death is not caused by activation of non-specific toxicity by over-expression of this transgene.

Recent studies have shown that autophagy and *Atg* genes are required for cell death in additional tissues during *Drosophila* development, including the ovary, embryonic serosal membrane, and intestine. In the ovary, developmental and nutrient deprivation-induced cell death requires *Atg* gene function[64-66]. Similarly, inhibition of autophagy by maintenance of cell growth inhibits serosal membrane cell death[67]. Autophagy also occurs in dying larval midgut cells[68], and *Atg* genes are required for clearance of this tissue[69].

It is not clear how autophagy regulates the distinct survival and death fates of cells. Autophagy may provide metabolic substrates to the dying cell to facilitate cell death, and studies of dying cells during cavitation of mouse embryonic stem cell embryoid bodies support this possibility[70]. Alternatively, autophagy may selectively degrade cell survival factors, and this possibility is supported by work in mammalian cell culture[71]. Significantly, a recent study indicates that the IAP Bruce is depleted by autophagy in dying nurse cells, and that this controls DNA fragmentation[66]. For this mechanism to be true, one must assume that autophagy is different in surviving and dying cells. Although little is known in this regard, a recent study shows that the engulfment receptor Draper is required

for autophagy in dying salivary gland cells, but not for autophagy that promotes cell survival in fatbody of starving *Drosophila* larvae[72].

Extensive autophagy may also deplete mitochondria and metabolic substrates, and this could lead to a metabolic catastrophe and death of cells. Although it is unclear how autophagy kills in most cells, this possibility is partly supported by the fact that excess autophagy that is induced by expression of the Atg1 protein kinase is sufficient to prematurely kill salivary gland cells in a caspase-independent manner[62]. Finally, it is possible that the combined activity of caspases and autophagy in cells is detrimental.

Relationship between autophagy and caspases

The expression of caspases, caspase regulators and *Atg* genes in dying larval salivary glands suggested the possibility that both caspases and autophagy may contribute to autophagic cell death[60,61]. Significantly, reduced function of either caspases or autophagy only partially inhibited salivary gland degradation, and the combined inhibition of caspases and loss of *Atg* gene function resulted in greater inhibition of salivary gland degradation than either of these genetic perturbations alone[62]. Similarly, both autophagy and caspases function in the death of neural stem cells in flies, as these cells live longer in animals with decreased function of both pro-apoptotic regulators and *Atg1* than when either autophagy or apoptosis regulators are impaired alone[73]. These data indicate that both caspases and autophagy contribute to the autophagic death of these cells, and suggest that a relationship may exist between these degradative processes.

Recent studies indicate that the relationship between caspases and autophagy may be context-specific in dying cells (figure 2). In salivary glands, these processes appear to function in parallel pathways; decreased caspase function fails to inhibit autophagy[21], and *Atg* mutations neither inhibit caspase activity nor lead to premature caspase activity[62]. By contrast, studies of cell death during oogenesis suggest that a hierarchical genetic relationship exists between caspases and autophagy (figure 2); the caspase DCP-1 and the IAP Bruce are required for autophagy during starvation-induced cell death[64]. However, Nezis et al. (2009 and 2010) present a different model for cell death during mid-oogenesis, as mutations in *Atg* genes result in the persistence of the IAP Bruce, reduced caspase activation and DNA fragmentation[65,66]. In addition, a recent study indicates that both autophagy and caspases function in the death of neural stem cells in flies, as these cells live longer in animals with decreased function of both pro-apoptotic regulators and *Atg1* than decreased when either autophagy or apoptosis regulators are impaired alone[73].

It is important to determine if autophagy kills cells, or is a cell clearance mechanism that functions either downstream or in parallel to caspases. A recent study indicates that autophagy plays a more prominent role than caspases in intestine cell death during fly development[69]. Although both caspase activity and autophagy are associated with the death of larval midgut cells[68,74], mutations in caspases and caspase regulators were not able to prevent destruction of these cells. By contrast, mutations in *Atg* genes inhibited degradation of midgut structures[69]. Although it is not clear how autophagy regulates the death of midgut cells, this is the first *in vivo* model where autophagy appears to regulate cell death in a caspase-independent manner. Therefore, future studies of larval midgut cell death in flies could significantly advance our understanding of how autophagy contributes to cell death.

Conclusions

Studies in *Drosophila* have made important contributions to our understanding of how cells die in the context of development. Here we have highlighted recent advances in fly cell

death research, including a better understanding of the mechanisms of apoptosis, as well as the demonstration of a role for autophagy in cell death. The strength of *Drosophila* as a model genetic system has enabled the investigation of the mechanisms underlying the death of cells under physiological conditions, but much remains to be learned. Additional genetic screens are needed to identify the genes that regulate these processes in cell specific contexts, as well as to fully understand how processes such as autophagy contribute to cell survival and death. Flies have also served as a useful model for diseases, and although the roles of different types of cell death in disease were not discussed here, such research in *Drosophila* should significantly advance medicine.

Acknowledgments

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Figure 1. Apoptosis in *Drosophila*

As in other organisms, caspases activation orchestrates apoptosis that involves cellular condensation, fragmentation and corpse engulfment. Caspases are made as inactive zymogens, but gain proteolytic activity after assembling into oligomeric complexes with its adaptor, Apaf1-related-killer. Recently, Tango7 was identified as another activator of caspases. In *Drosophila*, Inhibitors of Apoptosis Proteins (IAPs) play particularly visible roles in preventing caspase activation. Therefore, apoptosis requires the expression of IAP-antagonists, reaper, hid and grim. Phagocytes recognize apoptotic cells through surface proteins, Draper and Simu. Subsequently, a downstream signaling cascade that involves the kinase Shark and others involved in Ca^{2+} signaling are required to complete corpse engulfment.

Figure 2. Possible relationships between autophagy and caspases during autophagic cell death
 (A) Autophagy and caspases may function in a parallel genetic pathway, and this is supported by studies of fly salivary glands. (B) Autophagy and caspases may function in a common regulatory hierarchy. It is possible that either autophagy could regulate the activity of caspases, or caspases could regulate autophagy. Data to support these possible models have been provided in studies of fly ovaries.