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Seminars in Cell & Developmental Biology 16 (2005) 237–243

seminars in
CELL & DEVELOPMENTAL
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Review

# Mechanisms of steroid-triggered programmed cell death in *Drosophila*

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Available online 18 January 2005

**Abstract**

Studies in *Drosophila* have provided a detailed understanding of how programmed cell death is regulated by steroid hormones during development. This work has defined a two-step hormone-triggered regulatory cascade that results in the coordinate induction of central players in the death pathway, including the *reaper* and *hid* death activators, the *Apaf-1* ortholog *dark*, and the *dronc* apical caspase gene. Recent transcriptional profiling studies have identified many new players in this pathway. In addition, genetic studies are providing new insights into the control of autophagic cell death and revealing how this response is related to, but distinct from, apoptosis.


*Keywords:* Ecdysone; Autophagy; Gene regulation; Steroid hormones

**Contents**



## 1. Introduction

Although it is widely appreciated that programmed cell death must be precisely controlled during normal development, little is known about how this regulation is achieved. Small lipophilic hormones, including steroids, retinoic acid, and thyroid hormone, provide one of the best understood signals for directing the appropriate patterns of developmental cell death. In vertebrates, extensive studies have focused on how glucocorticoids trigger the destruction of excess thymocytes during T-cell maturation [1,2]. A more dramatic death response occurs in amphibians, where thyroid hormone triggers the massive destruction of the tadpole tail and remodeling of the intestine during metamorphosis [3–6]. Similarly, pulses of the steroid hormone ecdysone direct the stage-specific programmed cell death of obsolete larval tissues during *Drosophila* metamorphosis [7,8]. Studies of larval tissue histolysis in *Drosophila* have provided our clearest understanding of the molecular mechanisms by which hormones regulate cell death, due largely to the powerful combination of genetic and molecular tools provided by this organism. In this review, we focus on recent advances in understanding the molecular mechanisms of ecdysone-triggered programmed cell death. We discuss genomic efforts to identify hormone-

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1084-9521/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.semcdb.2004.12.007

regulated genes involved in this process, and how this system provides an ideal context for characterizing the genetic control of autophagic and apoptotic cell death responses.

## 2. Ecdysone directs stage-specific programmed cell death during metamorphosis

Ecdysone acts as a critical temporal signal in the insect, triggering each of the major developmental transitions in the life cycle [9]. The most dramatic of these responses occurs during metamorphosis, when two sequential pulses of ecdysone direct the transformation of a crawling larva into a highly mobile and sexually reproductive adult fly. A high titer pulse of ecdysone at the end of the third larval instar signals puparium formation, initiating metamorphosis and the prepupal stage in development (Fig. 1). This is followed by a second ecdysone pulse, approximately 10 h after puparium formation, that signals eversion of the adult head from the thoracic cavity, defining the prepupal-to-pupal transition (Fig. 1). Two divergent developmental programs are activated by ecdysone during metamorphosis—the massive destruction of obsolete larval tissues by programmed cell death and the simultaneous growth and differentiation of adult tissues from

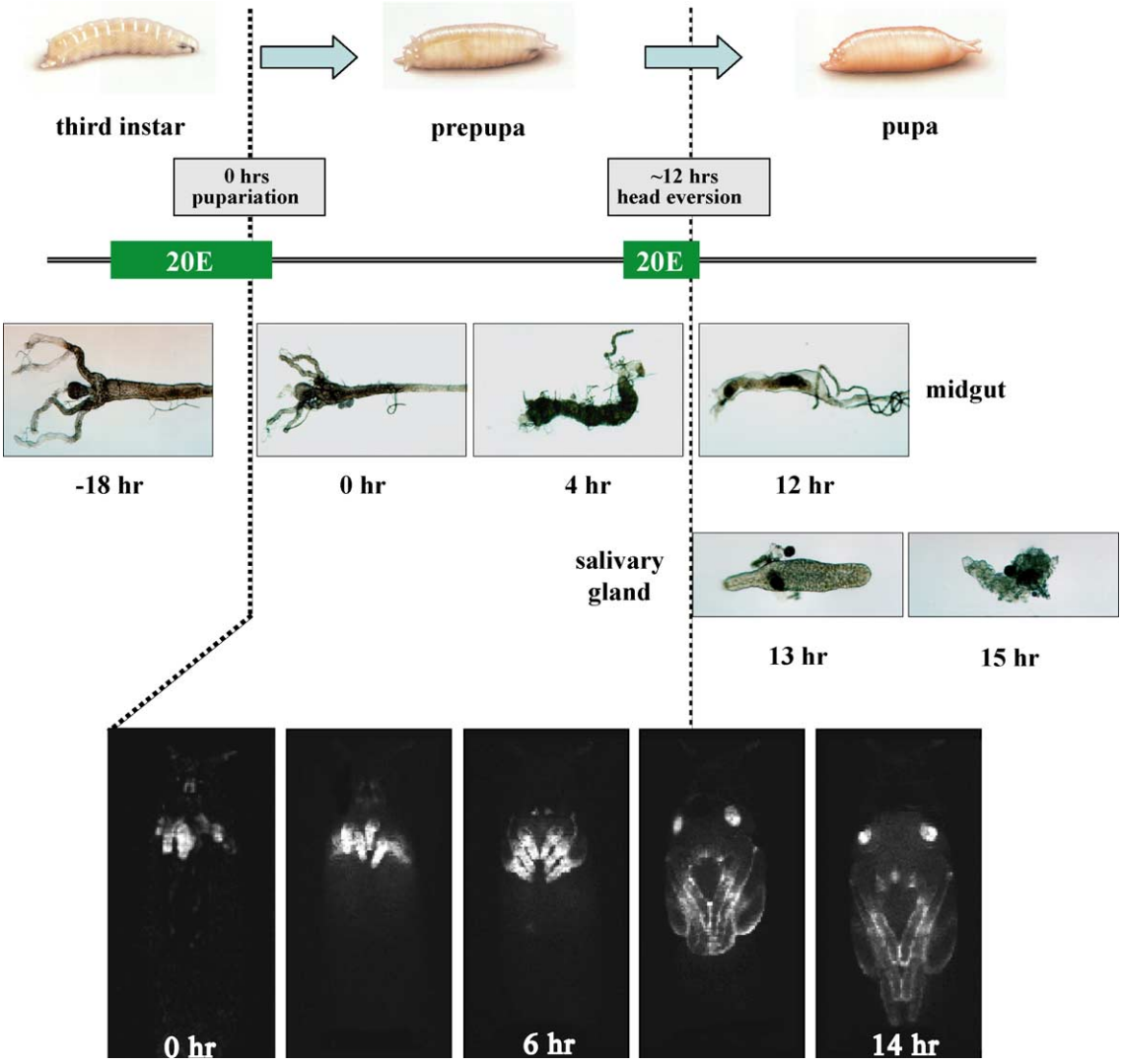


Fig. 1. Ecdysone triggers stage-specific biological responses during the onset of metamorphosis. Green boxes indicate the two ecdysone pulses that direct the onset of metamorphosis. The late larval ecdysone pulse triggers puparium formation, defining the larval-prepupal transition. This is followed ~10 h later by the prepupal ecdysone pulse that triggers adult head eversion at ~12 h, and the prepupal-pupal transition. The larval midgut begins to undergo cell death in newly formed prepupae, in response to the late larval ecdysone pulse. By 12 h after pupariation, the larval midgut cells have condensed to form the yellow body, surrounded by an intact adult gut that has developed from small clusters of progenitor cells. In contrast, the larval salivary glands survive the late larval ecdysone pulse but die rapidly following the prepupal pulse of ecdysone. Shown below the larval tissues are panels from a movie depicted imaginal disc development [10]. The adult legs elongate and evert, such that all six legs are arranged along the ventral surface of the animal by 6 h after pupariation. The wings are positioned laterally. Head eversion occurs at ~12 h after pupariation, along with leg and wind elongation, forming an immature adult fly by 14 h. The panels in this figure are adapted from Fig. 1 of Jiang et al. [8] and Fig. 6 of Ward et al. [10].

small clusters of progenitor cells [7,10]. Destruction of the larval tissues occurs in a precise stage-specific sequence, with the anterior muscles and larval midgut dying in response to the late larval ecdysone pulse, and the larval salivary glands and abdominal muscles dying in response to the subsequent prepupal ecdysone pulse [7,8] (Fig. 1). Studies of the genetic regulation of larval midgut and salivary gland histolysis have provided not only a model system for defining the molecular mechanisms of steroid-triggered cell death but also an ideal opportunity to determine how a repeated hormonal signal is refined into distinct stage-specific responses during development.

Programmed cell death is vital for the proper development of the *Drosophila* eye during the later stages of metamorphosis. The eye consists of about 750 functional units called ommatidia, each with eight photoreceptor neurons and six support cells, separated by interommatidial pigment cells. Approximately, a third of the pigment cells are destroyed during metamorphosis through precise patterns of cell death, serving to sculpt the final structure of the eye [11]. This death occurs uniformly across the eye field, over the course of a few hours starting at 24 h after puparium formation, in synchrony with a high titer pulse of ecdysone [9]. Although no clear ties have been made between pigment cell death and ecdysone, it would be interesting to determine if the hormone acts as a temporal cue for this response.

## 3. Ecdysone triggers programmed cell death via a two-step transcriptional cascade

Like vertebrate hormones, ecdysone exerts its effects via members of the nuclear receptor superfamily that act as ligand-regulated transcription factors. The ecdysone receptor is a heterodimer of two nuclear receptors, the EcR ecdysone receptor and Ultraspiracle (USP), the fly retinoid X receptor ortholog [12]. The ecdysone/EcR/USP complex directly regulates primary-response gene expression, including the induction of a small set of transcription factor-encoding early genes that act to transduce and amplify the hormonal signal by directly regulating late secondary-response target genes [13–15]. The early genes include the *Broad Complex* (*BR-C*), which encodes a family of zinc finger transcription factors, the *E74* ETS-domain transcription factor gene, and *E93*, which encodes a novel site-specific DNA binding protein. Whereas the *BR-C* and *E74* are induced in response to each ecdysone pulse during development, *E93* is more specific in its response to the hormone, displaying stage-specific expression in the larval salivary glands, foreshadowing the onset of programmed cell death [16].

In contrast to the balance between death activators and death repressors that determines the precise patterns of cellular apoptosis, destruction of the larval tissues during *Drosophila* metamorphosis results from a major transcriptional switch in which multiple pro-apoptotic genes are coordinately induced by ecdysone. Immediately prior to the onset of midgut and salivary gland cell death, ecdysone induces the death activator genes *reaper* (*rpr*) and *head involution defective* (*hid*), the caspase gene *dronc*, the *Apaf-1* ortholog *dark*, and the *CD36* homolog *croquemort* (*crq*), a marker for phagocytes [8,17–20]. This transcriptional response appears to represent a terminal commitment to cell death, ensuring the rapid and massive destruction of these tissues.

Ecdysone is both necessary and sufficient to trigger programmed cell death in the larval midgut and salivary glands [8,21]. Genetic studies indicate that ecdysone-induced *rpr* and *hid* expression play a critical role in this response. A null mutation in *rpr* results in no defects in larval tissue cell death, while reduction of *hid* function by RNAi leads to partially penetrant salivary gland death defects [22,23]. Loss-of-function for both *rpr* and *hid*, however, results in highly penetrant larval midgut and salivary gland death defects, indicating that these genes act in a redundant manner to direct the destruction of larval tissues. Moreover, low levels of *diap1* expression are required throughout larval stages to prevent premature larval tissue cell death, indicating that as in embryos, *rpr* and *hid* trigger cell death by overcoming the inhibitory effect of DIAP1 [23]. No studies have yet addressed functional roles for *dark* and *crq* in larval tissue cell death. Two recent papers, however, have described the phenotypes of *dronc* mutants, providing the first genetic study of caspase function during metamorphosis [24,25]. *Dronc* mutants progress through larval development but arrest during pupal stages, having successfully made the prepupal-to-pupal transition. Mutations in this CARD-containing apical caspase result in persistent salivary glands with defects in caspase activation and DNA fragmentation [24].

Interestingly, a similar regulatory pathway appears to be operative during later stages of metamorphosis, when select groups of neurons in the central nervous system undergo programmed cell death in response to the decreasing titer of ecdysone [26]. The death activators *rpr* and *grim* (but not *hid*) are induced immediately before the death of these neurons, and this induction is dependent upon the fall in hormone titer [27,28]. In addition, death of a select group of peptidergic neurons are sensitive to the dose of *rpr* and *grim*, implicating their functional role in this death response [28]. Taken together with the data from larval tissue cell death during the onset of metamorphosis, these studies suggest that ecdysone directs death responses at multiple stages of the life cycle through the coordinate induction of pairs of death activator genes.

The critical roles for ecdysone-induced expression of *rpr*, *hid* and *dronc* in larval tissue cell death raises the question of how these death activators are controlled by the hormone. Extensive studies have been directed at making this regulatory link in the larval salivary glands, focusing on *EcR*, *BR-C*, *E74*, and *E93*, based on the observation that each of these genes is required for salivary gland cell death [17,19,29,30]. Ecdysone directly induces *rpr* and *dronc* expression through an EcR/USP response element in their promoters [17,31]. Studies in tissue culture cells suggest that this effect is medi-

ated by an EcR-interacting factor, the *Drosophila* homolog of the arginine-histone methyltransferase CARM1, which appears to be required for maximal death gene transcription [32]. The direct induction of *rpr* and *dronc* by ecdysone is supplemented by a secondary-response to the hormone, in which *BR-C* is required for *rpr*, *hid*, and *dronc* transcription, and *E74* is required for maximal levels of *hid* expression [17,30,33]. *BR-C* appears to directly regulate *dronc* transcription as mutagenesis of *BR-C* binding sites within the *dronc* promoter leads to significantly reduced promoter activity in transfected tissue culture cells [34]. *E93* mutants display the most global defects in cell death gene expression, with reduced levels of *rpr*, *hid*, *dronc*, and *crq* [19,30]. The observation that *BR-C* and *E74* expression is also reduced in this mutant, however, raises the question of whether these effects on death gene transcription are direct or indirect. Studies of the DNA binding specificity of E93 should provide insights into this question. Curiously, *dark* is not significantly affected by mutations in *BR-C*, *E74*, or *E93*, although its expression is induced in salivary glands immediately before the onset of death [30]. It is possible that *dark* induction is mediated by other transcription factors that are expressed in doomed larval salivary glands, such as Bunched, Dif, or Dfos [18,20,35]. Collectively these studies demonstrate that ecdysone directs the destruction of larval tissues through a two-step regulatory hierarchy in which early ecdysone-induced transcription factors coordinate the induction of key death activators (Fig. 2).

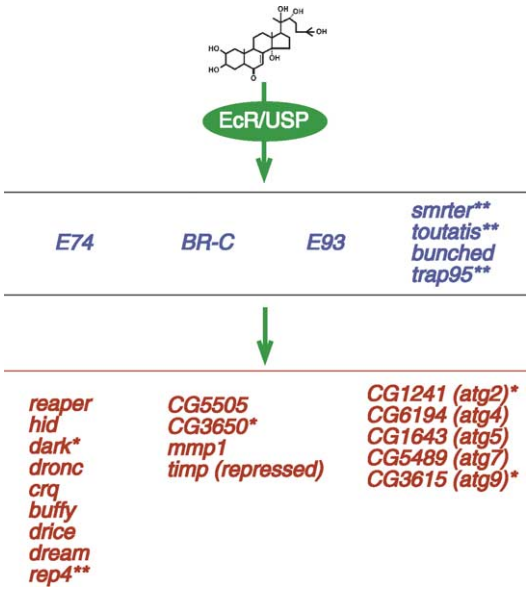


Fig. 2. An ecdysone-triggered genetic cascade directs larval salivary gland cell death. The ecdysone/EcR/USP complex directly induces primary-response gene expression, including the *E74*, *BR-C*, and *E93* transcription factor genes. Additional transcriptional regulators that are up-regulated preceding salivary gland cell death are depicted at the top in blue [18,20]. Secondary-response genes are depicted below in red, including known death regulators on the left, noncaspase proteases in the center, and homologs of yeast ATG genes on the right [18,20]. The secondary-response genes have all been shown to be affected by *E74*, *BR-C*, and/or *E93* mutations, with the exception of those with a single asterisk [20]. Those with double asterisks were not examined in mutant salivary glands. All genes are up-regulated in dying salivary glands except *timp*, which is repressed.

## 4. Microarray and SAGE studies reveal novel potential regulators of steroid-triggered cell death

Recent microarray and SAGE studies have significantly expanded our understanding of the transcriptional response to ecdysone in doomed larval salivary glands, identifying several hundred transcripts that are either up-regulated or down-regulated immediately preceding cell death [18,20]. Many of these new target genes comprise functional groups that are consistent with roles in cell death, including transcription factors, death regulators, cell remodeling genes, and noncaspase proteases (Fig. 2). Both studies detected induction of known players in the salivary gland death response, including *E93*, *rpr*, *hid*, *dark*, and *crq*, thus validating their experimental design [18,20]. In addition, a number of chromatin modifying factors and transcription co-factors are up-regulated, including the chromatin remodeling gene *toutatis* (*tou*), the *smrter* nuclear receptor co-factor gene, and *trap95*, which encodes a predicted component of the transcription mediator complex defined by its physical association with thyroid hormone receptor in mammals [18,20] (Fig. 2). Several transcription factors were also found to be up-regulated in doomed larval salivary glands, including *bunched* (*bun*) and *CG5465*, which is predicted to encode a zinc finger protein. These observations reinforce the notion that larval tissue destruction is determined by a major transcriptional commitment to cell death.

Several classes of genes with defined roles in cell death were detected as up-regulated in these profiling studies, including the cell remodeling gene *cut up* (*ctp*), the pro-apoptotic Bcl-2 family member *buffy*, and the caspase-encoding genes *drice* and *dream* [18,20] (Fig. 2). Lee et al. [20] also detected transcriptional induction of *rep4*, which encodes an apparent caspase-activated DNase that could contribute to DNA fragmentation during cell death. Although many cellular changes are controlled by caspases, a subset of dynamic cytoskeletal cell changes are unaffected by p35 expression, suggesting that noncaspase proteases have important roles in programmed cell death [36]. Hence, it is intriguing that the *mmp1* matrix metalloproteinase gene is highly induced in dying salivary glands, while the *timp* metalloprotease inhibitor gene is repressed [18,20]. Genetic studies have shown that *mmp1* is required for tissue remodeling during development and *timp* is sufficient to inhibit *mmp* function, indicating that these genes could contribute to larval tissue histolysis [37]. Consistent with the dramatic cellular changes in dying tissues, genes associated with cellular remodeling were also found to be significantly induced. These include the myosin ATPase gene *crinkled* (*ck*) and members of the Rho/Rac family of small GTPases [20].

In an effort to integrate some of these genes into the ecdysone regulatory cascades, Gorski et al. [18] quantitated

the levels of 18-up-regulated genes in both wild type and *E93* mutant salivary glands by RT-PCR, and determined that all but one were dependent on *E93* function for their maximal expression. These genes include a number with unknown function as well as two cecropin genes involved in the microbial defense response pathway, *CecA1* and *CecB*, the *Doa* protein kinase gene, *mmp1*, the mitochondrial carrier protein gene *CG5254*, and the proposed protein tyrosine phosphatase gene *Ptpmeg*. A more comprehensive analysis was performed by Lee et al. [20] who analyzed the expression of 21 target genes by northern blot hybridization using staged salivary glands from wild type, *BR-C*, *E74*, and *E93* mutants. This study significantly expanded our understanding of the regulatory interactions that coordinate steroid-triggered death gene expression (Fig. 2). Thus, for example, *buffy* and *drice* are both reduced in *E74* and *E93* mutant salivary glands, *dream* is affected by all three mutations, and *bun* and *mmp1* are dependent on *BR-C* and *E93*. Collectively these microarray and SAGE studies better define the dramatic transcriptional reprogramming of gene expression that accompanies salivary gland cell death, and provide a strong foundation for future reverse genetic studies of target gene function.

## 5. Apoptotic and autophagic cell death

Morphological studies by Kerr et al. [38], combined with more recent efforts [39], have defined apoptosis as a cell death response that involves nuclear condensation, membrane blebbing, DNA fragmentation, and caspase activation. Larval midgut and salivary gland cell death is accompanied by the formation of acidic autophagic vesicles in the cytoplasm, defining these as autophagic cell death responses [40,41]. Whereas isolated cells are targeted for death by apoptosis, autophagic cell death is usually observed when entire tissues are destroyed during development. Although the early morphological studies relegated apoptosis and autophagy to distinct forms of cell death, recent studies of *Drosophila* salivary gland histolysis have demonstrated that these two processes also have much in common.

Electron microscopic images of dying salivary glands reveal a large number of autophagosomes that contain cellular components, such as mitochondria and Golgi apparatus, a defining feature of autophagic death [40]. These tissues, however, also score positive for caspase activation, DNA degradation, and acridine orange staining, all hallmarks of apoptosis [8,36]. In addition, whereas apoptotic cells are engulfed by macrophages that express CRQ, this CD36 receptor homolog is expressed within dying salivary glands, suggesting that both the death and degradation machinery is active in tissues that die by autophagy. In an effort to dissect the regulation of autophagic cell death, Lee and Baehrecke [40] have examined salivary glands from *BR-C*, *E74*, and *E93* mutants. Consistent with a central role for *E93* in ecdysone-regulated death gene transcription, *E93* mutant salivary gland cells display a lack of autophagic vesicles and caspase activation, arresting early in the death response [36,40]. At least part of this effect could go through *dronc*, a target gene of *E93*, as *dronc* mutant salivary gland cells lack autophagosomes [24]. Additionally, mutations in *E74* and *BR-C* result in persistent salivary glands with defects in caspase activation but normal signs of autophagy, apparently due to a late arrest in the death program [36,40]. These studies define *E93* as a key determinant of autophagic cell death and provide a foundation for using genetics to tease apart the regulation of apoptosis and autophagy in the destruction of larval tissues during metamorphosis.

Autophagy was originally defined in yeast as a non-selective process of cytoplasmic degradation wherein amino acids and other nutrients are recycled from proteins and organelles within the cell [42]. This response requires ~20 autophagy-related genes, or ATG genes, that direct lysosomes to recycle unneeded cytoplasmic material during periods of starvation. Interestingly, *Drosophila* homologs of many of these ATG genes are induced immediately before salivary gland cell death, with the expression of some of these genes dependent on early gene function [18,20] (Fig. 2). In addition, a recent study has shown that autophagy is induced by ecdysone in the larval fat body at the onset of *Drosophila* metamorphosis, apparently to mobilize nutrients during the period of starvation that follows the cessation of larval feeding [43]. These observations raise the interesting possibility that autophagy and apoptosis serve distinct functions during larval tissue cell death. Induction of autophagy would allow the pupa to fully exploit the nutrients within dying tissues, supporting the growth and differentiation of new adult structures. Subsequent induction of cell death would direct the destruction of these doomed larval tissues. Several lines of evidence support this model. Autophagic cell death and nutrient recycling are both induced by ecdysone in *Drosophila*, and are characterized by the formation of autophagosomes [40,43]. Expression of the p35 caspase inhibitor is sufficient to suppress salivary gland degradation and DNA fragmentation but does not prevent the formation of autophagic vesicles [40]. Similarly, the phenotypes of *BR-C* and *E74* mutant salivary glands suggest roles for these regulatory genes in the induction of apoptosis but not autophagy. Key to testing this model is determining whether the ATG genes that are up-regulated in dying salivary glands play a functional role in nutrient recycling and/or cell death. A hint in this direction is provided by a recent study which shows that mammalian ATG7 and another ATG gene, *beclin1* (*Atg6*), are required for programmed cell death induced by caspase 8 inhibition in tissue culture cells [44]. It will be interesting to determine whether *Drosophila* ATG genes display similar roles in cell death during development.

## 6. Common and unique aspects of larval midgut and salivary gland cell death

A small ring of adult progenitor cells is located at the anterior end of the larval salivary glands, adjacent to the

ducts that lead to the esophagus [7]. These cells divide and differentiate during pupal development to form the adult salivary glands. In contrast, the larval midgut cells are surrounded by small islands of imaginal cells that are committed to form the future adult gut. This close physical arrangement of larval and adult progenitor cells in the midgut suggests that larval cell death and adult differentiation may be coordinated in this tissue. Larval midgut cell death occurs with classic hallmarks of autophagic cell death, including the formation of vesicles that contain cellular organelles, as well as DNA fragmentation, acridine orange staining, and caspase activation [8,41]. The late larval ecdysone pulse induces a transcriptional cascade in the larval midgut that is similar to that seen in the salivary glands [41]. Genetic studies, however, have revealed distinct differences. Mutations in *BR-C* and *E93* lead to different defects in larval midgut cell death, while *E74* mutations have no effect on the destruction of this tissue. Interestingly, no defects are seen in development of the adult midgut or in the shortening of the larval midgut that occurs during early prepupal stages in *BR-C*, *E74*, or *E93* mutants, suggesting that midgut shortening is a direct consequence of adult gut formation. Like dying salivary glands, *rpr*, *hid*, *dark*, *crq* and *dronc* are co-ordinately induced during larval midgut cell death; however, these genes are subject to distinct regulation. A mutation in *BR-C* results in an absence of *rpr*, *hid*, and *crq* expression, while *E93* mutants only display defects in *dronc* transcription [41]. Unlike the salivary glands, where a reduction in *hid* function results in partially penetrant death defects, both *hid* and *rpr* must be disrupted before death defects can be detected in the larval midguts, indicating that these death activators are fully functionally redundant in this tissue [23]. Similarly, *dronc* mutants display no defects in larval midgut cell death [24]. Given that midgut cell death can be blocked by expression of the p35 caspase inhibitor, another caspase must contribute to the destruction of this tissue during metamorphosis [8].

The roles of metalloproteases in programmed cell death were first explored during *Xenopus* metamorphosis, identifying a critical role in intestinal remodeling [45]. Recent genetic studies in *Drosophila* have revealed a similar function for the *mmp2* matrix metalloprotease gene in larval midgut cell death [37]. Whereas wild type larval midguts undergo dramatic condensation and cell death by 4 h after puparium formation, the midguts of *mmp2* mutants fail to die and persist into pupal stages. Future genetic studies that compare the programmed cell death of the larval midguts and salivary glands should not only shed light on how these responses are regulated in a distinct stage-specific manner by ecdysone, but also provide insights into how their unique arrangements of larval and adult cells might impact their destruction during metamorphosis.

## Acknowledgements

V.P.Y. is supported by an NIH Developmental Biology Training Grant (5T32 HD07491). Research in our lab is supported by the NIH and the Howard Hughes Medical Institute.

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