Steroid-triggered death by autophagy

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Summary

Programmed cell death is a critical part of normal development, removing obsolete tissues or cells and sculpting body parts to assume their appropriate form and function. Most programmed cell death occurs by apoptosis of individual cells or autophagy of groups of cells. Although these pathways have distinct morphological characteristics, they also have a number of features in common, suggesting some overlap in their regulation. A recent paper by Lee and Baehrecke provides further support for this proposal.[1] These authors present, for the first time, a genetic analysis of autophagy, using the steroid-triggered metamorphosis of *Drosophila* as a model system. They demonstrate a remarkable degree of overlap between the control of apoptosis and autophagy as well as a key role for the steroid-inducible gene *E93* in directing the autophagic death response. This paper also shows that *E93* can direct cell death independently from the known death-inducer genes, defining a novel death pathway in *Drosophila*. *BioEssays* 23:677–682, 2001. © 2001 John Wiley & Sons, Inc.
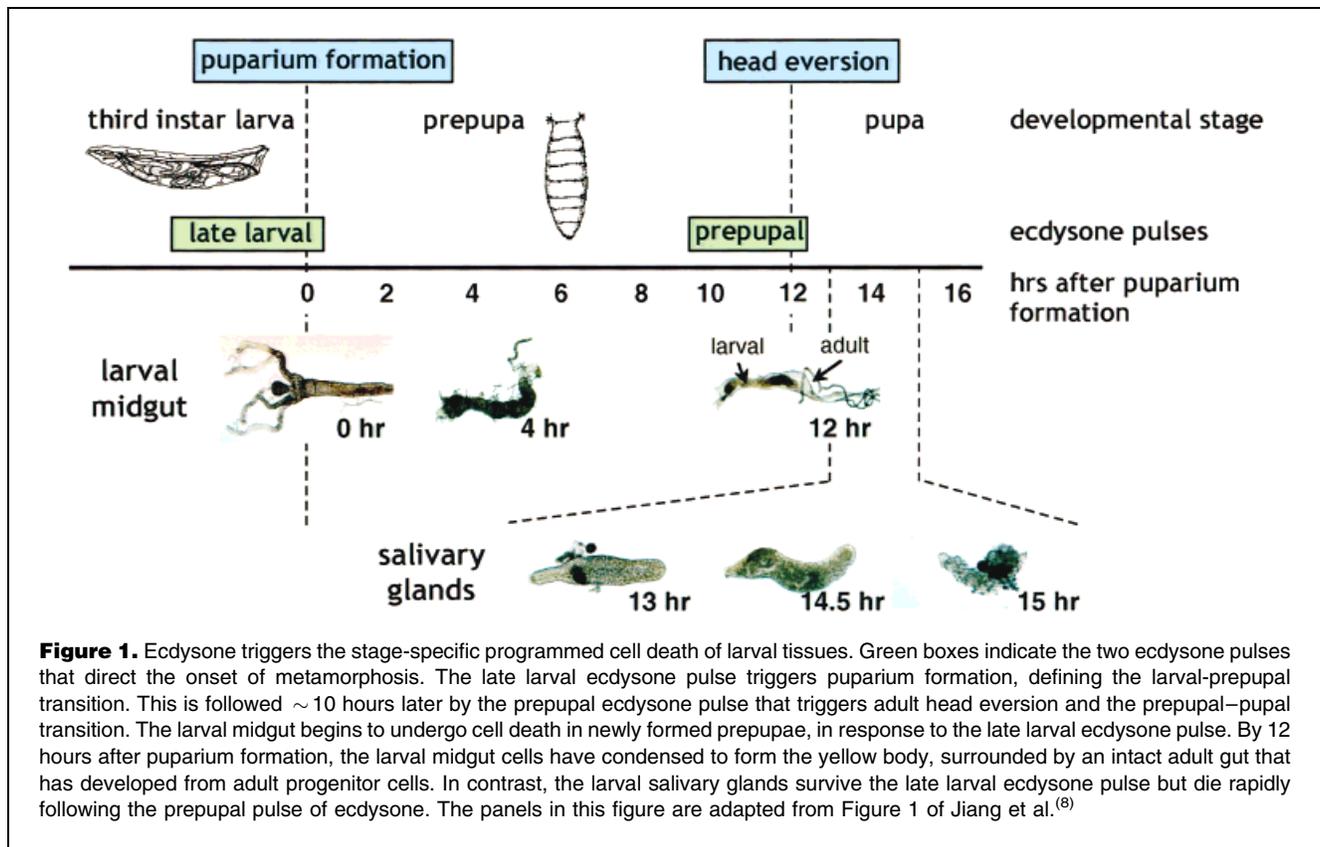
Introduction

Intensive studies over the past few decades have focused on understanding the regulation of programmed cell death during normal development as well as its misregulation in human disease. These efforts have been primarily directed at apoptosis, a form of programmed cell death that is usually seen in individual dying cells. As described by Kerr and colleagues,[2] apoptosis is characterized by initial condensation of the nucleus and cytoplasm followed by DNA fragmentation and eventual elimination of the dying cell by phagocytes. What is not as widely appreciated, however, is that a morphologically distinct form of programmed cell death, called autophagy, is at least as prevalent during development. This form of cell death is seen when entire tissues, or parts thereof, are committed to destruction. As described by Schweichel and Merker,[3] autophagy occurs via the formation of multiple acidic autophagic vacuoles within the doomed cells. This is accompanied by a wide range of changes in organelle morphology as well as changes in plasma membrane structure. The nucleus can become condensed, although this is usually not as dramatic as that seen in apoptosis, followed by massive cellular degeneration.

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The key distinction between these two death pathways is that cells undergoing apoptosis are degraded by the lysozymes of a second, phagocytic cell, while autophagic cell death is true suicide, where the cell is destroyed by its own lysosomal contents. This fundamental difference might lead one to expect that apoptosis and autophagy are under very different regulation; however, it is clear that some dying cells display features of both pathways.[4,5] Moreover, relatively little is known about the regulation of autophagy, in sharp contrast to the extensive studies that have been aimed at characterizing apoptosis. This disparity in our understanding, however, is rapidly changing, due largely to studies of a dramatic example of autophagy — the destruction of larval tissues during insect metamorphosis. A recent paper by Lee and Baehrecke takes advantage of this system to provide insight into the regulation of autophagic cell death, and identifies a key role for the *E93* gene in controlling this response.[1]

Steroid regulation of programmed cell death during *Drosophila* metamorphosis

Sequential pulses of the steroid hormone 20-hydroxyecdysone (hereafter referred to as ecdysone) act as critical regulators of the *Drosophila* life cycle, signaling each of the major developmental transitions.[6] At the end of the third larval instar, a high titer ecdysone pulse triggers puparium formation, initiating metamorphosis and the prepupal stage in development (Fig. 1). This is followed by a second ecdysone pulse, approximately 10 hours after puparium formation, which signals eversion of the adult head and defines the prepupal-to-pupal transition. In response to these two ecdysone pulses, the obsolete larval tissues undergo rapid and massive programmed cell death to be replaced by adult tissues and structures that differentiate from small clusters of predetermined progenitor cells.[7] The larval midgut and salivary glands illustrate the stage-specificity of these death responses, with the midgut initiating cell death in early prepupae and the larval salivary glands dying in early pupae, immediately after the prepupal pulse of ecdysone (Fig 1).[8] Salivary gland cell death exhibits hallmark features of autophagy, including increases in acid phosphatase activity and increased number and size of autophagic lysosomal vaculoles.[9,10] These observations have been confirmed by Lee and Baehrecke.[1] In addition, Lee and Baehrecke move beyond morphological criteria to ask if salivary gland

Figure 1. Ecdysone triggers the stage-specific programmed cell death of larval tissues. 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 $\sim$10 hours later by the prepupal ecdysone pulse that triggers adult head eversion 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 hours after puparium formation, the larval midgut cells have condensed to form the yellow body, surrounded by an intact adult gut that has developed from adult progenitor cells. In contrast, the larval salivary glands survive the late larval ecdysone pulse but die rapidly following the prepupal pulse of ecdysone. The panels in this figure are adapted from Figure 1 of Jiang et al.[8]

autophagic cell death has any molecular machinery in common with apoptosis — and find that it indeed does.

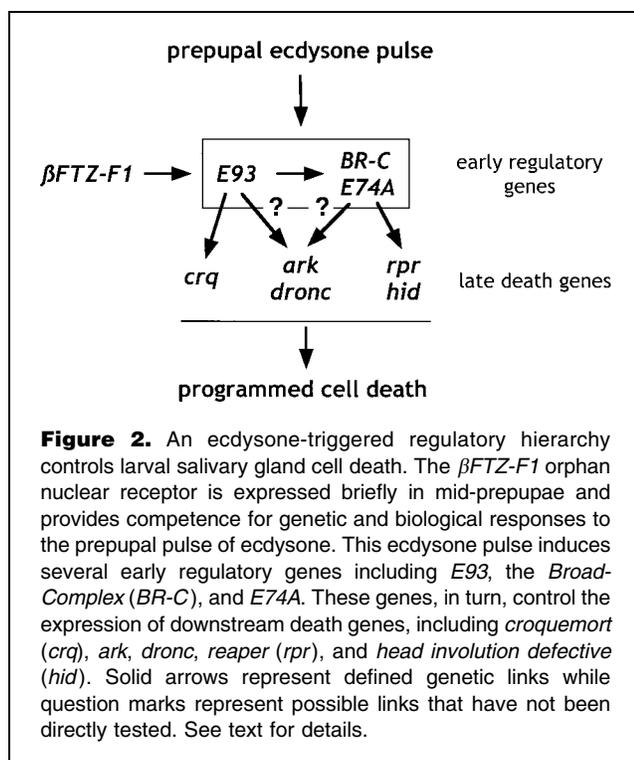## Caspase activity is required for salivary gland autophagic cell death

Lee and Baehrecke ask first if caspase activity is required for the destruction of larval salivary glands by autophagy. They show that expression of the baculovirus caspase inhibitor p35 results in persistent glands that fail to degenerate. Closer examination of these glands revealed that most (89%) progress normally to a late stage in autophagy, although DNA fragmentation fails to occur. The remaining salivary glands (11%) were blocked at an early stage of autophagy with large autophagic vacuoles and intact plasma membranes. Increasing the levels of p35 expression did not increase the proportion of glands displaying this phenotype, indicating that p35 levels are not limiting. Thus, although it is clear that caspases are required for autophagic destruction of the salivary glands, the role of caspases in distinct stages of autophagy remains unclear. This conclusion contrasts with current models that propose no role for caspase activation in autophagic cell death.[5] The partial phenotype observed by Lee and Baehrecke indicates, however, that we have more to

learn about the multiple roles of caspases in the execution of programmed cell death responses.

An ecdysone-triggered genetic cascade directs larval salivary gland cell death

Ecdysone exerts its effects on *Drosophila* development by triggering stage- and tissue-specific regulatory hierarchies. The hormone directly induces a small set of early regulatory genes that activate larger sets of downstream secondary-response late genes.[11] These target genes, in turn, control the appropriate biological response to each ecdysone pulse during development.

Four ecdysone-inducible regulatory genes have been identified that are required for larval salivary gland cell death – *βFTZ-F1*, the *Broad-Complex (BR-C), E74A*, and *E93*. The *βFTZ-F1* orphan nuclear receptor is expressed for a brief interval in mid-prepupae and functions as a critical competence factor for both genetic and biological responses to the prepupal ecdysone pulse, including larval salivary gland cell death.[12,13] In the presence of this competence factor, the prepupal ecdysone pulse induces maximal levels of *BR-C, E74A*, and *E93* early gene expression (Fig. 2). Both the BR-C zinc finger proteins and the E74A ETS domain transcription

Figure 2. An ecdysone-triggered regulatory hierarchy controls larval salivary gland cell death. The βFTZ-F1 orphan nuclear receptor is expressed briefly in mid-prepupae and provides competence for genetic and biological responses to the prepupal pulse of ecdysone. This ecdysone pulse induces several early regulatory genes including *E93*, the *Broad-Complex* (*BR-C*), and *E74A*. These genes, in turn, control the expression of downstream death genes, including *croquemort* (*crq*), *ark*, *dronc*, *reaper* (*rpr*), and *head involution defective* (*hid*). Solid arrows represent defined genetic links while question marks represent possible links that have not been directly tested. See text for details.

factor are widely expressed in response to ecdysone pulses during development, and contribute to multiple biological pathways including larval salivary gland cell death.[14–17] In contrast, *E93* is induced in a highly stage- and tissue-specific manner during development, foreshadowing the death of larval tissues during the onset of metamorphosis.[18] Consistent with this expression pattern, *E93* is necessary for the destruction of larval midguts and salivary glands in response to ecdysone.[19] The *E93* protein sequence provides no clues regarding its function, although *E93* protein is localized in the nucleus and bound to specific sites on the salivary gland polytene chromosomes suggesting that it may directly control gene expression.

Genetic and molecular studies in *Drosophila* have identified homologs of most of the genes involved in mammalian and *C. elegans* programmed cell death.[20–23] These include an APAF-1/CED-4 homolog, *ark*,[24–26] the ecdysone-inducible caspase *dronc*[27], and two key death-inducer genes, *reaper* (*rpr*) and *head involution defective* (*hid*).[28,29] The *Df(3L)H99* deletion that removes both *rpr* and *hid*, along with a related death inducer, *grim*, results in a complete block in embryonic programmed cell death.[29,30] In addition, ectopic expression of either *rpr*, *hid* or *grim* is sufficient to trigger cell death, arguing that these genes are central players in the cell death pathway.[28,30,31] Recent studies have shown that *rpr, hid*, and *grim* exert their effect by binding to IAPs, preventing IAP association with caspases and thereby allowing the caspase

cascade to initiate apoptosis.[32–34] The vertebrate gene SMAC/DIABLO performs a similar role in triggering the death response, and thus is likely to represent a functional homolog of *rpr*, *hid*, and *grim*.[35] Finally, *croquemort* (*crq*), a CD36 receptor homolog, is expressed in macrophages that engulf apoptotic corpses during *Drosophila* embryonic development. This gene is required for engulfment and is regulated by the amount of apoptosis that is occurring in the organism.[36]

Interestingly, *ark*, *dronc*, *rpr*, *hid*, and *crq* are all induced in larval salivary glands immediately before the onset of cell death, implicating a direct role in this response (Fig. 2).[19] Moreover, genetic studies have linked the expression of these death genes to the activity of *E93*, *BR-C*, and *E74A*.[15,19] *E93* is required for the induction of all five death genes, the *BR-C* is required for *rpr* and *hid* induction, and *E74A* is required for maximal *hid* expression. These interactions have defined an ecdysone-triggered genetic cascade that directs the stage-specific destruction of the larval salivary glands during *Drosophila* metamorphosis (Fig. 2).

Ecdysone-inducible early genes act at different stages of salivary gland cell death

Earlier studies had shown that mutations in βFTZ-F1, *E93*, *BR-C*, and *E74A* result in defects in larval salivary gland cell death.[12,15,17,19] Lee and Baehrecke confirm and extend these observations by showing that these mutations result in blocks at two distinct stages in autophagy. Both βFTZ-F1 and *E93* mutant salivary glands are arrested before the onset of cell death, containing large autophagic vacuoles, normal plasma membranes, and intact nuclei. In contrast, *BR-C* and *E74A* mutant salivary glands have some cells in the process of autophagy, although the nuclei remain largely intact. It thus appears that βFTZ-F1 and *E93* work early in the death response, and that the *BR-C* and *E74A* execute later events in this pathway. This observation is consistent with earlier studies of βFTZ-F1 that establish this gene as a key regulator of all responses to the prepupal pulse of ecdysone, including salivary gland cell death. For *E93*, this upstream function is consistent with the reduced levels of *BR-C* and *E74A* transcription that are seen in *E93* mutant salivary glands.[19] However, rather than simply invoke an epistatic relationship between these early genes, Lee and Baehrecke propose a more intriguing and complex model. They speculate that *E93* acts more directly on the early destruction of vacuoles and plasma membranes, features normally associated with autophagy, while the *BR-C* and *E74A* act later in the death pathway.

E93 is a novel death inducer that acts independently of *rpr, hid,* and *grim*

Evidence in support of a more direct role for *E93* in autophagy derives from a series of elegant gain-of-function studies, in which Lee and Baehrecke show that *E93* is sufficient to trigger

many aspects of cell death. Interestingly, these responses fall into two categories — those that are dependent on *rpr, hid*, and *grim*, and those that are independent of these death genes.

Lee and Baehrecke ectopically express *E93* during embryogenesis and show that this results in rapid and widespread cell death as indicated by an increase in acridine orange staining, Nile Blue staining, the formation of apoptotic corpses, engulfment by macrophages and, finally, cellular elimination. These effects are seen in cells expressing *E93*, indicating that this is a cell autonomous death response. The authors also performed this study in a *Df(3L)H99* genetic background, thereby eliminating any potential contribution from *rpr*, *hid*, and/or *grim*. Remarkably, many aspects of *E93*-driven cell death still occur in this genotype, defining *E93* as a novel death inducer that can function independently of *rpr, hid*, and *grim*. This sets *E93* cell death apart from other *Drosophila* apoptotic responses that depend on integrity of the H99 genetic interval.[29] The only known exception to this is nurse cell apoptosis during oogenesis, where cell death occurs normally in the absence of *rpr, hid*, and *grim*.[37] Efforts are currently underway to determine whether *E93* might contribute to this exceptional death response (Buszczak and Cooley, personal communication).

Lee and Baehrecke show that, in the absence of *rpr, hid*, and *grim*, ectopic *E93* expression in embryos can still result in the formation of apoptotic corpses, engulfment by macrophages, and cellular elimination. However, nuclear changes associated with cell death — DNA fragmentation, acridine orange staining, and Nile Blue staining — all fail to occur. Thus, although *E93* is sufficient to trigger a death response that has characteristics of both apoptosis and autophagy, only the nuclear apoptotic responses require the activity of *rpr, hid*, and/or *grim*. The possible role of the *BR-C* and *E74A* in regulating nuclear apoptotic responses, however, remains unclear. In the salivary glands, this is complicated by the fact that maximal *BR-C* and *E74A* expression are dependent on *E93* function.[19] Further studies will be required to elucidate the specific contributions of these regulators to directing the different steps in larval salivary gland cell death.

A role for croquemort in autophagy directed by E93

The ability of *E93* to direct phagocytic engulfment of apoptotic corpses raised the possibility that this gene might induce *crq* expression, which is known to be required for phagocytosis during normal embryonic cell death.[36] Lee and Baehrecke show that this is indeed the case. Ectopic *E93* is sufficient to induce *crq* transcription in embryos, and the levels of *crq* mRNA are significantly reduced in *E93* mutant salivary glands. Moreover, *E93*-induced *crq* expression is not reduced by the elimination of *rpr, hid* and *grim*, which are normally required for *crq* expression during embryonic cell death. Thus, once again, *E93* is sufficient to direct a key aspect of the apoptotic pathway

in the absence of the classic *Drosophila* death inducer genes. In addition, Lee and Baehrecke detect *crq* protein in the cytoplasm of wild-type dying salivary gland cells, further indicating that *crq* function is not restricted to apoptosis. Rather, it appears that *crq* is also acting during autophagic cell death, possibly as a direct response to *E93*. Moreover, the pattern of *crq* expression provides a molecular marker for apoptosis or autophagy, where *crq* is expressed independently of the dying cell in apoptosis and within the dying cell during autophagy.

Apoptosis and autophagy—variations on the theme of cell death

Lee and Baehrecke have made a significant contribution to our understanding of programmed cell death by using genetics to study its regulation in the context of an intact developing organism. Importantly, they show that *E93* is a novel death inducer that can function independently of the classic *rpr/hid/grim* pathway. Moreover, they demonstrate that *E93* is not only required for the autophagic destruction of larval salivary glands during metamorphosis but is also sufficient to trigger hallmark features of apoptosis in embryos, indicating that these two pathways share common regulatory mechanisms. Because cell death in *Drosophila*, as in vertebrates, can occur by either apoptosis or autophagy, it is clear that further genetic studies in flies will provide a better understanding of the common and distinct levels of control that determine the nature of a programmed cell death response.

The work of Lee and Baehrecke also sets the stage for more detailed studies of larval tissue autophagy in *Drosophila*. One question that emerges from this study is the role of caspases in the autophagic death response. It remains unclear why most glands are able to proceed to a late stage in autophagy in the presence of p35. In addition, further studies will be required to tease out the complex combinatorial interactions between *E93*, the *BR-C* and *E74A* transcription factors, and the downstream genes that execute the death program (Fig. 2). Finally, it will be interesting to determine the mechanisms of *E93* action. Although E93 protein binds to specific sites in the salivary gland polytene chromosomes, it lacks any known DNA-binding domains.[18] The presence of two LXXLL motifs within its coding region, however, raises the interesting possibility that E93 may act as a nuclear receptor co-factor and thus indirectly regulate gene expression through interactions with proteins such as βFTZ-F1 or the ecdysone receptor.[38] The identification of *crq* as a possible direct target of *E93* regulation should facilitate our understanding of its mode of action.

Lee and Baehrecke's genetic study also has implications for a wide range of autophagic cell death responses that have been identified in other organisms, including tail degeneration during amphibian metamorphosis, intersegmental muscle death in *Manduca sexta*, and posterior silk gland destruction

in *Bombyx mori*.[4,5] The dependence of tail regression on thyroid hormone indicates that autophagic responses are not restricted to ecdysone-triggered responses in insects, but can occur in response to vertebrate hormones as well. Moreover, death of the APR(6) motoneuron by autophagy during *Manduca* metamorphosis indicates that individual cells can display this death response.[39] This observation is consistent with the ability of *E93* to direct hallmarks of apoptosis in embryos and autophagy in larval salivary glands, arguing that cellular context may be just as important as the genetic program for determining whether a death response will occur by an apoptotic or autophagic death pathway.

Finally, this paper by Lee and Baehrecke provides further evidence that studies of derived cells maintained in culture may not provide an accurate indication of how programmed cell death is regulated in the intact animal. For example, the classic model for studying steroid-triggered programmed cell death is the elimination of vertebrate thymocytes in response to glucocorticoids.[40] Yet, these cells do not undergo autophagy, which is the predominant cell death response induced by hormones during normal development.[4,40] Similarly, little is known about the extracellular triggers that induce cell death at the appropriate time and place during development, leading to the widely accepted use of chemicals and growth factor withdrawal as a means of inducing a death response. It seems likely that further studies of programmed cell death triggered by natural inducers, such as steroids and thyroid hormone, combined with classic genetic approaches, will provide a more accurate indication of how cell death is controlled in the context of an intact developing organism.

Acknowledgments

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