

12 Programmed Cell Death in Insects

Susan E Fahrbach

Department of Biology, Wake Forest University,
Winston-Salem, NC, USA

John R Nambu

Department of Biological Sciences,
Charles E. Schmidt College of Science,
Florida Atlantic University, Boca Raton, FL, USA

Lawrence M Schwartz

Department of Biology, 221 Morrill Science Center,
University of Massachusetts, Amherst, MA, USA

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Summary

Programmed cell death (PCD) is a normal component of development and homeostasis in animals, plants, and even some single-celled organisms. While there appear to be multiple forms of PCD, the best characterized are apoptosis and autophagy. In insects, PCD has been observed in diverse tissues, and is required for the normal completion of metamorphosis. This chapter reviews the history of studies of PCD in two key models, the hawkmoth *Manduca sexta* and the fruit fly *Drosophila melanogaster*. This highly active field of research is built on a sturdy foundation of decades of studies of hormonally-regulated PCD

in neuromuscular systems in these two species. Major discoveries based on insect research include identification of the RHG protein apoptosis activators and IAP family proteins as well as the first demonstration of the role of ubiquitination in muscle PCD. Contemporary studies of PCD in neuromuscular systems and dying larval tissues (salivary gland, midgut) have demonstrated the co-occurrence of apoptotic and autophagic gene expression in individual cells fated to die. The study of PCD during metamorphosis in insects is a mature field of inquiry that offers numerous opportunities for study of mechanisms related both to insect development and human disease.

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12.1. Introduction

Programmed cell death (PCD) is a fundamental component of the post-embryonic development of insects, and has been the subject of at least 3000 PubMed-indexed publications as of this writing. PCD is particularly evident during metamorphosis of the neuromuscular systems of holometabolous insects, and these systems will be emphasized in this chapter. Nerve and muscle cells born during embryogenesis are deleted in a segment- and cell-specific fashion at both the larval–pupal and pupal–adult transitions. Although such deaths presumably occur in all insects with complete metamorphosis, almost all of the information on this topic has been obtained from two species: the fruit fly *Drosophila melanogaster* (Diptera: Drosophilidae), and the tobacco hawkmoth *Manduca sexta* (Lepidoptera: Sphingidae). The sophistication of the genetic tools and genomic resources available in *Drosophila* has allowed many key regulatory pathways in insect PCD to be identified. In addition, the success of large-scale genetic screens for embryonic pattern formation as well as detailed analysis of *Hox* gene clusters have provided insight into mechanisms underlying elaboration of body axes, specification of cell populations, and organogenesis. These insights into early development provide a strong base for an understanding of metamorphosis that is now supplemented by large-scale analyses of gene expression using tools such as DNA micro- and tiling arrays. The small size of fruit flies, however, limits the utility of this species for physiological and biochemical studies involving hormone manipulations, tissue transplantation, and surgical interventions. The lepidopteran *Manduca sexta* is an alternative model uniquely suited for these experimental approaches, serving as the insect equivalent of the laboratory white rat (Fahrback, 1997). Experimental results from these species provide the foundation of this chapter, but the explosion in availability of sequenced insect genomes that characterized the first decade of the 21st century opens the door to true comparative studies of PCD.

12.2. PCD, Apoptosis, Autophagy, or Necrosis?

It is now recognized that cell death is a normal part of life in animals, plants, and even some single-celled organisms (Ameisen, 2002; Segovia *et al.*, 2003; Reape and McCabe, 2008; Shemarova, 2010). For example, parasitic protists may undergo PCD to minimize their impact on the immune system of a mammalian host (Deponte, 2008), and several types of bacteria exhibit PCD in response to stress or nutrient deprivation (Engelberg-Kulka *et al.*, 2006). In multicellular organisms, the capacity to remove selected cells during development provides organisms

with a plastic response to developmental contingencies. In vertebrates, PCD has been demonstrated to:

- 1 Match sizes of interacting populations of cells, such as oligodendrocytes, to the axons they myelinate (Barres *et al.*, 1992).
- 2 Remove deleterious cells, such as self-reactive T cells (Smith *et al.*, 1989; Opferman, 2008).
- 3 Sculpt the body, such as in the loss of interdigital cells in the limb bud of the developing embryo (Zuzarte-Luís and Hurlé, 2002; Montero and Hurlé, 2010).
- 4 Remove developmentally obsolete cells, such as the tail of the tadpole and other larval organs in amphibians (Yoshizato, 1996; Ishizuya-Oka *et al.*, 2010).

In insects, dramatic examples of PCD take place during embryogenesis and during metamorphosis, when larval tissues are destroyed to allow the formation of new adult structures. The predictable events of insect metamorphosis provide accessible experimental systems for defining the regulatory mechanisms that mediate PCD, and which can be extended to other organisms.

12.2.1. Apoptosis

The best-characterized morphology associated with both developmental and pathological cell death is apoptosis, a term coined by Kerr, Currie, and Wyllie (Kerr *et al.*, 1972). At its introduction, apoptosis was a morphological term that implied neither mechanism nor specific developmental context. During the process of apoptosis, cells shrink and display plasma membrane zeosis, defined as the formation of numerous protuberances or blebs. Time-lapse photographs of cells undergoing apoptosis in culture look like drops of water skittering on a hot skillet. Under *in vivo* conditions, apoptotic cells are typically rapidly phagocytosed either by neighboring cells or by phagocytes (Franc, 2002; Geske *et al.*, 2002; Hart *et al.*, 2008). An informative video is available at: <http://www.youtube.com/watch?v=V-NsR-krKME&feature=related>.

The nucleus of apoptotic cells condenses, and the chromatin becomes electron-dense and margined along the inner aspect of the nuclear envelope. These morphological changes are physical manifestations of a massive cleavage of genomic DNA that occurs when endogenous nucleases become activated and cleave the linker DNA between individual nucleosomes (Wyllie *et al.*, 1984; Enari *et al.*, 1998). The fragmentation of the genome can be visualized when DNA is extracted and fractionated by size in agarose (Eastman, 1995). It is sometimes difficult to detect clear apoptotic ladders because, in many tissues, dying cells are intermingled with healthy ones. In addition, because tissues are homogenized during DNA isolation, it is impossible to determine which subpopulation

of cells contributed the degraded DNA. An alternative strategy for detecting fragmented genomic DNA is to employ *in situ* labeling techniques on tissue sections, such as terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling, referred to as the TUNEL method (Gavrieli *et al.*, 1992; Ben-Sasson *et al.*, 1995). The TUNEL method is now supplemented by other *in situ* methods, including use of monoclonal antibodies to detect single-stranded DNA (Frankfurt *et al.*, 1996). Other *in situ* methods rely on antibodies targeted to proteins that form the molecular machinery of apoptosis or to cleavage products resulting from the action of proteolytic enzymes on their protein targets during the execution phase of apoptosis (Huerta *et al.*, 2007). Antibodies that recognize activated, cleaved caspase-3 can be used to detect apoptosis in *Drosophila* (Xu *et al.*, 2006; Shapiro *et al.*, 2008), although there has been some controversy regarding the identity of endogenous proteins recognized by these antisera (Fan and Bergmann, 2010). New computational tools have been developed for high throughput analysis of apoptotic cells *in vivo*, which will facilitate genetic screens (Forero *et al.*, 2009).

12.2.2. Autophagy

While apoptosis is the morphology most commonly observed during PCD, particularly during embryogenesis, it is not the only one. Other suicidal cell deaths are designated autophagic (Bursch, 2001; Ryoo and Baehrecke, 2010). Like apoptosis, autophagy appears to be an ancient cellular process with counterparts in unicellular organisms (King and Gottlieb, 2009). Cells undergoing autophagy lack, in general, initial condensation of chromatin, and instead form autophagic bodies (autophagosomes) – double-membrane bounded portions of cytoplasm that eventually fuse with a lysosome (Xie and Klionsky, 2007). The identification and analysis of autophagosomes was originally dependent upon transmission electron microscopy, and there was a lag in development of useful cellular markers for autophagic cells. This lag initially hindered analysis of the developmental significance of autophagy.

One of the most common triggers for autophagy is nutrient limitation (King and Gottlieb, 2009), and in this context autophagy represents a cell survival mechanism (because the net effect is to recycle cellular materials into metabolic pathways) rather than a mediator of PCD. However, autophagy plays an active role in PCD in several tissues in developing *Drosophila*, including the salivary gland (Berry and Baehrecke, 2007) and ovary (Nezis *et al.*, 2009). There is evidence for cross-talk between autophagic and apoptotic death pathways (Hou *et al.*, 2008; Nezis *et al.*, 2010).

Recent interest in the contribution of autophagy to human diseases such as cancer, Crohn's disease, and neurodegeneration associated with aging has led to development

of several novel markers for autophagy, including homologs of yeast genes essential for autophagy, such as LC3 in mice and *Draut1* in *Drosophila melanogaster* (Juhász *et al.*, 2003; Tanida *et al.*, 2008). As a result, rapid progress in understanding autophagy in insects can be expected (Barth *et al.*, 2010).

12.2.3. PCD is an Inclusive Term

In this chapter, we employ the term PCD to describe cell loss that occurs in a temporally and spatially predictable manner. PCD encompasses both apoptotic and autophagic cell deaths that occur as normal components of development. Current evidence argues strongly that, even within a single cell, multiple molecular mechanisms of PCD can be simultaneously active (Schwartz *et al.*, 1993a; Jones and Schwartz, 2001; Thummel, 2001; Muppidi *et al.*, 2004; Stoica and Faden, 2010). For example, death of the larval salivary glands during metamorphosis in *Drosophila* requires a combination of apoptosis and autophagy, both of which are activated by pulses of the steroid 20-hydroxyecdysone (20E) secreted by the ring gland at pupation (Jiang *et al.*, 2000; Berry and Baehrecke, 2007; Conradt, 2009). A trend in the current literature is to refer to specific examples of PCD in terms of the underlying biochemical pathways (e.g., caspase-dependent, autophagy (Atg)-dependent) when these have been experimentally determined.

12.2.4. Necrosis

Apoptosis and autophagy are orchestrated developmental decisions, but all cells can be induced to die by necrosis, a form of cell death that occurs in response to external insult such as heat, salt, abrasion, toxin exposure, etc. (Dive *et al.*, 1992; Kroemer *et al.* 1998). Necrosis typically follows disruption of plasma membrane integrity, which in turn results in an influx of water and ions, most notably calcium, which leads to subsequent cellular swelling and lysis. In vertebrates, which have an adaptive immune response, necrosis provides a valuable warning to the immune system that focal injury has occurred, because the cellular constituents that are liberated during necrosis are highly inflammatory (MacDonald and Stoodley, 1998; Fadok *et al.*, 2001). Endogenous responses to necrosis are responsible for much of the secondary tissue damage that accompanies injury (Whelan *et al.*, 2010).

12.2.5. Hybrid Forms of Cell Death

Novel cell death pathways, including hybrids that incorporate features of established pathways, have been described, but their relevance to insect systems in general, and insect neuromuscular systems in particular, has not yet been established. A recent example from mammalian cells is

necroptosis, an orderly, programmed form of necrosis. Necroptosis can be triggered in cells lacking caspase activity by a death domain containing kinase RIP1 (Christoferson and Yuan, 2010) as a consequence of the binding of TNF (tumor necrotic factor) to its receptor, TNFR1 (Vandenabeele *et al.*, 2010). It is associated with ischemic cell death following stroke (Degterev *et al.*, 2005). One lesson from the broader cell death literature for investigators of PCD in insects is that cross-talk across death/survival pathways is to be expected. One consequence of such cross-talk is that simple, tried and true histological or single marker methods are not always reliable gauges of cellular status (Mohseni *et al.*, 2009).

12.3. Historical Overview and Current Trends

The first morphological description of PCD during insect metamorphosis we have found in the literature is a description of the death of intersegmental muscles (ISMs) in the silkworm *Bombyx mori* (Kuwana, 1936). The phenomenon of ISM death during insect metamorphosis was largely unknown outside of Asia until independently described by Finlayson, who also studied development in Lepidoptera (Finlayson, 1956). With the exception of a modest number of descriptive papers documenting examples of PCD in other insect taxa, little was published about cell loss during arthropod development for most of the subsequent decade. A compendium of the early studies of cell death in insects can be found in Glücksmann's early review of the field (Glücksmann, 1951). A comprehensive investigation of PCD based on analysis of the ISMs was initiated in the mid-1960s by Lockshin and Williams (1964, 1965a, 1965b, 1965c). In fact, it was in R. A. Lockshin's 1963 doctoral dissertation on ISM development that the term programmed cell death was first introduced.

The first description of neuronal death during insect metamorphosis detailed the post-embryonic changes in neuronal populations observed in the abdominal ganglia of *Manduca sexta* (Taylor and Truman, 1974). Truman and his colleagues exploited their ability to count motoneurons in individual ganglia by backfilling cut ends of peripheral nerves with cobalt; they reported that motoneurons were lost from the abdominal ganglia at both the larval–pupal and pupal–adult transitions. Many of these motoneurons were later determined to be uniquely identifiable cells, which permitted the precisely regulated nature of metamorphic neuron death to be established (Truman, 1983; Levine and Truman, 1985).

Studies by Schwartz and Truman subsequently demonstrated that changes associated with ISMs at the end of metamorphosis are controlled by hormonal signals, particularly by the steroid 20E (Schwartz and Truman, 1982; 1983). The principle of steroid control of nerve and

muscle fate at metamorphic transitions was extended to the death of abdominal neurons that occurs after adult eclosion in *Manduca* (Truman and Schwartz, 1984), and to the death of *Manduca* proleg motoneurons (and their associated muscles) at pupation (Weeks and Truman, 1985; Weeks, 1987, 1999). Using the ISMs from the moth *Manduca sexta*, Schwartz and colleagues were the first to clone death-associated transcripts from any organism (Schwartz *et al.*, 1990a). The results of these influential studies are detailed below.

Descriptive studies of the death of neurons and muscles after adult eclosion in *Drosophila melanogaster* (Kimura and Truman, 1990) foreshadowed the initiation in the 1990s of an intensive genetic analysis of PCD in fruit flies. Efforts focused on identifying the molecular mechanisms that mediate PCD, and the extracellular signals that trigger it. Subsequently, interest grew in the development of *Drosophila* models of human neurodegenerative disease (e.g., Mutsuddi and Nambu, 1998; Driscoll and Gerstbrein, 2003; Lu, 2009). The underpinning of this work is the consensus that mechanisms of cell death are broadly conserved across phylogeny. Studies of PCD in insects are well positioned to address specific questions concerning metamorphosis, basic questions in cell biology, and topics of relevance to human health.

12.3.1. Earlier Reviews of PCD Relevant to Insects

The field of PCD in insects has been extensively reviewed, although many reviews focus on narrow subtopics within the field. For early reviews, see Wing and Nambu (1998), Abrams (1999), Bangs and White (2000), and Lee and Baehrecke (2000). Recent reviews covering the discovery of the first genes associated with PCD in *Drosophila melanogaster* include those by Hay and Guo (2006); Steller (2008); and Xu *et al.* (2009). Autophagic and apoptotic cell deaths during *Drosophila* metamorphosis were first compared by Thummel (2001). Notable reviews of PCD during animal development in general (including insects) were provided by Jacobson *et al.* (1997), Milligan and Schwartz (1997), Meier *et al.* (2000), and Baehrecke (2002). Kornbluth and White (2005) and Conradt (2009) compared the molecular basis of PCD during development in three model organisms: the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and the mouse *Mus musculus*.

12.3.2. Current Trends in PCD Research and Publication

Persistent research interest in the general field of cell death studies is indicated by the success of print and online journals dedicated to PCD. These include *Cell Death and Differentiation*, *Autophagy*, *Journal of Cell Death*, *Cell Death*

and Disease, and Apoptosis, all of which publish studies based on insect models.

12.4. The *Manduca* Model

12.4.1. Choice of *Manduca sexta* as a Model System for the Study of PCD of Nerve and Muscle Cells

Insect endocrinologists and neurobiologists favor *Manduca sexta* because of its large size and ease of rearing in culture on an artificial diet (Bell and Joachim, 1976; Arnett, 1993; Fahrbach, 1997). This species has a facultative rather than an obligatory diapause, and therefore all life stages can be produced in the laboratory at any time of the year. Development from egg to adult requires roughly 40 days. There are five larval stages (each referred to as an instar, so that the final larval stage is the fifth instar) and a single pupal stage. The adult emerges after about 18 days, and lives approximately 10 days. The large size of this insect permits extensive surgical and endocrine manipulations. The central nervous system (CNS) consists of a dorsal brain and a ventral nerve cord. The abdominal portion of the ventral nerve cord retains its segmental organization of discrete thoracic and abdominal ganglia throughout the post-embryonic period – a feature that facilitates anatomical, electrophysiological, and functional analyses.

The thoracic and abdominal musculature of *Manduca sexta* has been described (Eaton, 1988). Many aspects of neuromuscular metamorphosis first described in *Manduca* were subsequently identified in metamorphosing fruit flies (e.g., Kimura and Truman, 1990; Truman *et al.*, 1994), despite evidence that the insect orders of Lepidoptera and Diptera have been evolving independently for at least 200, and possibly 300, million years (Hoy, 2003). This conservation suggests that many aspects of neuromuscular metamorphosis in insects, including PCD, are ancient in origin. This relationship permits phenomena first observed in *Manduca* to be subjected to molecular genetic analysis by shifting to *Drosophila* for follow-up studies (e.g., Robinow *et al.*, 1993). Conversely, orthologs of genes first identified in *Drosophila* can be cloned in *Manduca*, and their patterns of expression followed throughout development in identified neurons and muscles (e.g., Nagy *et al.*, 1991; Kraft and Jäckle, 1994).

The striking changes in the organization of the nervous and muscular systems that accompany lepidopteran metamorphosis result from a combination of post-embryonic cell proliferation, modification of structures formed initially in the embryo, and PCD. In the CNS, while a large proportion of developing neurons and glia undergo PCD, these deaths occur in specific, isolated cells. In contrast, in the developing musculature there is wholesale loss of entire bundles of muscle fibers (Schwartz, 2008). The coordinated loss of the ISMs following adult eclosion in

Manduca offers a robust system for the study of PCD, because these cells are exceptionally large, easily accessible throughout the entire process of degeneration, and uncontaminated by persisting muscle fibers (Figure 1).

The ISMs of *Manduca* are composed of giant syncytial fibers approximately 5 mm long and 1 mm in diameter. These embryonically-derived fibers are used by the larva for locomotion, and by the pupa for defensive and respiratory behaviors. The ISMs also perform the major abdominal movements required for the eclosion behavior of the adult moth. The ISMs begin to atrophy 3 days prior to adult eclosion. This period of atrophy results in a loss of 40% of muscle mass; despite this atrophy, the ability to contract is maintained during this early phase (Schwartz and Ruff, 2002). Once the ISMs have participated in eclosion, they are no longer required for any adult behavior. They die over the course of the subsequent 30 hours (Lockshin and Williams, 1965a; Schwartz, 2008).

12.4.2. Hormonal Regulation of Metamorphosis

As in all insects, metamorphosis in *Manduca* is regulated by two categories of developmental hormones: ecdysteroids and juvenoids (reviewed by Nijhout, 1994; Truman and Riddiford 2002). The ecdysteroids are steroid hormones that exert their primary actions through members of the nuclear receptor superfamily of proteins (Robinson-Rechavi *et al.*, 2003). The juvenile hormones are terpinoids (Riddiford, 2008). At present, the cellular mode of the action of the juvenile hormones in metamorphosis remains to be defined, and likely involves more than one signaling pathway (Gilbert *et al.*, 2000; Jones and Jones, 2000; Wilson *et al.*, 2006; Bitra and Palli, 2009). Experiments on the hormonal regulation of neuromuscular metamorphosis in *Manduca* have clearly established that the changes in cell populations and connectivity that occur during neuromuscular metamorphosis are controlled by direct actions of ecdysteroids and juvenile hormones on neurons, glial cells, and muscle (Bennett and Truman 1985; Streichert *et al.*, 1997). Receptors for ecdysteroids have been localized to neuronal, glial cell, and muscle cell nuclei in *Manduca* (Bidmon and Koolman, 1989; Fahrbach and Truman, 1989; Bidmon and Sliter, 1990; Fahrbach, 1992; Hegstrom *et al.*, 1998).

12.4.3. PCD of Neurons during Metamorphosis

12.4.3.1. Background and overview Neuronal death during metamorphosis in *Manduca* involves motoneurons, interneurons, and identified peptidergic neurons. Most studies have focused on the death of motoneurons not only because of the greater ease of identification of specific neurons across individuals, but also because the highly visible degeneration of muscles during post-embryonic development often suggests that innervating

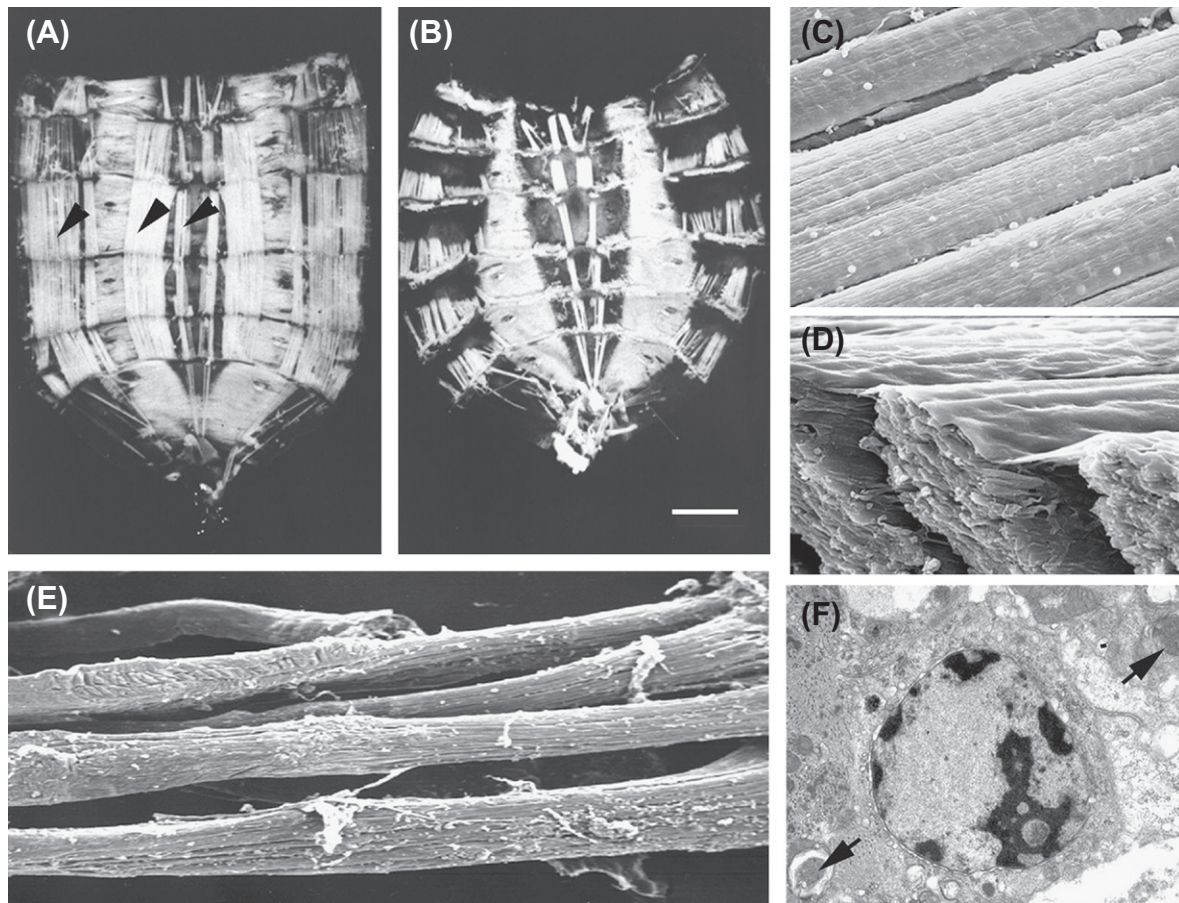


Figure 1 The morphology of the ISMs at the light (A, B), scanning electron microscopic (C–E), and transmission electron microscopic (F) levels. ISMs were examined in the abdomens of newly eclosed adults (A) and 30-hour-old adults (B). Three sets of bilaterally symmetric ISMs span four abdominal segments (arrowheads) in the newly eclosed adult, but they disappear during the subsequent 30 hours. Other abdominal muscle groups are spared, and persist throughout adulthood. The ISMs are composed of large, well-defined muscle fibers (C, D) that rapidly lose mass as they die (E). This death is cell autonomous, and the dying fibers are not phagocytosed (F). Ultrastructural analysis of the ISMs during death reveals pyknotic nuclei and numerous autophagic vesicles (arrows) (F). Scale bar approximately 5 mm (A, B), 10 μ m (C), 4 μ m (D), 40 μ m (E), and 2 μ m (F). Adapted from Schwartz (2008).

motoneurons will be lost, so that investigators in effect “know where to look.” The presence of sexually dimorphic structures in the adult, such as the oviduct (Giebultowicz and Truman, 1984; Thorn and Truman, 1989) and brain (Kalberer *et al.*, 2010), also often suggests where neuronal PCD will be found. Dying peptidergic neurons can be identified after the detection of stage-specific patterns of antibody labeling (Ewer *et al.*, 1998). Cell counts and the presence of small pyknotic (shrunken) profiles often provide the only anatomical clues that interneurons have died. The observation that the corpses of many dying neurons are not immediately phagocytosed and therefore persist in the ganglia facilitates the detection of PCD well after the initiation of the PCD program.

Patterns of neuronal death during metamorphosis in *Manduca* have been most fully described for the motoneurons of the abdominal ganglia, although histological surveys of thoracic ganglia reveal that PCD of neurons is

also found in this tissue at the metamorphic transitions (S. E. Fahrbach, unpublished data) (Figure 2). At the larval–pupal transition, the best-studied examples of dying neurons are the proleg motoneurons (Weeks and Truman, 1985; Weeks, 1999). Studies of neuronal death associated with adult eclosion have focused primarily on the death of motoneurons (including the death of proleg motoneurons that persist after pupation) in the unfused abdominal ganglia, A3 through A5. In addition to the deaths of fully differentiated neurons, PCD of undifferentiated neurons occurs in the imaginal nests of the segmental ganglia, both as the larvae feed and at the onset of metamorphosis (Giebultowicz and Truman, 1984; Booker and Truman 1987; Thorn and Truman, 1989; Booker *et al.*, 1996). PCD of neuroblasts that are active during post-embryonic life terminate proliferation in specific lineages in both the segmental ganglia and the brain: in *Manduca*, the death of neuroblasts has been most comprehensively studied in

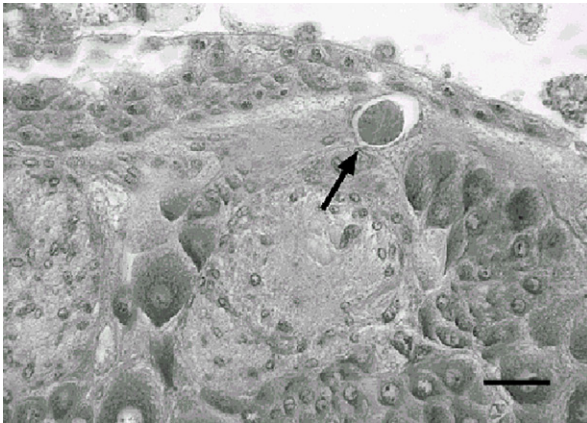


Figure 2 Transverse sections (8 μm) through the first thoracic (prothoracic or T1) ganglion of the ventral nerve cord of *Manduca sexta* fixed in alcoholic Bouins 72 hours after pupal ecdysis. The ganglion was dehydrated, embedded in a paraffin-based medium, sectioned, and stained with hematoxylin and eosin. The arrow points to the shrunken, condensed profile of a motoneuron that has undergone PCD. Because of the absence of phagocytosis in the ventral nerve cord during metamorphosis, the persisting fragments of such neurons are easily detected in insect nervous tissue prepared using routine histological techniques. Scale bar = 30 μm .

the segmental ganglia (Booker and Truman, 1987; Booker *et al.*, 1996).

The emphasis on PCD of neurons at the larval–pupal and pupal–adult transitions in *Manduca* should not obscure the fact that the majority of larval neurons survive metamorphosis and become integral components of the adult nervous system.

12.4.3.2. PCD of abdominal motoneurons during metamorphosis in *Manduca sexta* With rare exceptions, the abdominal motoneurons of *Manduca* are born during embryonic life. At both the larval–pupal and the pupal–adult metamorphic transitions, some of the larval motoneurons lose their muscle targets as a consequence of changes in the organization of the skeletal musculature (Levine and Truman, 1985). At the larval–pupal transition, some of these now targetless motoneurons die, including motoneurons that innervate muscles associated with the five pairs of abdominal prolegs, the locomotory appendages of the caterpillar that disappear at pupation (Weeks and Truman, 1985). In some segments, however, the proleg motoneurons survive this round of PCD, and, after a period of dendritic regression, acquire new muscle targets in the developing adult (Weeks and Ernst-Utzschneider, 1989; Weeks *et al.*, 1992). Targetless motoneurons therefore can either undergo PCD or become respecified. The death of these motoneurons appears to be triggered by endocrine cues independent of contact with target muscles (Bennett and Truman, 1985; Weeks and Truman, 1985). Abdominal motoneurons that lose their muscle targets after adult eclosion all die within 48 hours

of emergence; possibly the short (approximately 10-day) life of the adult may not provide sufficient opportunity for respecification. It should be noted that unpublished studies on the giant silkworm *Antheraea pernyi* (J. W. Truman) and *Hyalophora cecropia* (S. E. Fahrbach) suggest that, in some species, motoneurons persist after the death of the ISMs, neither respecifying nor dying. The survival of these motoneurons therefore is not dependent on target-derived trophic signals, in contrast to vertebrate motoneurons. Comparative studies on a broader range of insect taxa are needed to complete our understanding of neuronal PCD following adult eclosion.

12.4.3.3. Sex-specific PCD of abdominal motoneurons during metamorphosis The populations of abdominal motoneurons are the same in male and female larvae, but during metamorphosis the genital segments undergo sex-specific morphological changes that are accompanied by a wave of sex-specific PCD in the ganglia that innervate these segments (Giebultowicz and Truman, 1984). This is an example of equal opportunity sexual differentiation, as some motoneurons persist in males but degenerate in females, while others show the opposite pattern (Thorn and Truman, 1989). There is a sex difference in the timing of sex-specific PCD relative to pupation, with most neurons in females dying during the first 2 days following pupation, while most neurons in males die during the third to the sixth days after pupation (Thorn and Truman, 1994a).

An interesting example of sex-specific neuronal death in *Manduca* is the case of the imaginal midline neurons (IMNs). These are unusual motoneurons that are born post-embryonically during the fourth (penultimate) larval instar. These motoneurons innervate visceral rather than skeletal musculature, and a subset can be tracked during metamorphosis because they are immunoreactive with an antibody against molluscan small cardioactive peptide b (Thorn and Truman, 1994b). IMNs that innervate the sperm duct in males are absent from the terminal ganglia of females, while IMNs that innervate the oviduct in females are absent from the terminal ganglia of males. There is some evidence that contact with an appropriate target enhances the survival of the IMNs that innervate the sperm duct in males, another atypical (but vertebrate-like) aspect of their physiology.

12.4.3.4. PCD of identified peptidergic neurons during metamorphosis Two identified interneurons (INs) that contain crustacean cardioactive peptide (CCAP), cell 27 and IN 704, undergo PCD within 36 hours of adult eclosion (Ewer *et al.*, 1998). Both cell 27 and IN 704 display increases in cGMP immunoreactivity during larval ecdyses. Cell 27 also shows this response at pupal ecdysis and adult eclosion (Ewer and Truman, 1997). Because application of CCAP to the isolated CNS can trigger the motor patterns of ecdysis, this set of neurons appears to be

central to the control of molting behavior (Gammie and Truman, 1997; Mesce and Fahrbach, 2002). The death of peptidergic neurons involved in the control of ecdysis behavior, after the moth's molting career is finished, supports the hypothesis that obsolescent neurons are actively eliminated from the *Manduca* nervous system.

12.4.3.5. PCD in the brain during metamorphosis

Detailed studies of specific regions of the *Manduca* brain have clearly demonstrated that newly-generated neurons die during adult development. PCD is a prominent feature of the development of both the medulla and lamina cortices in the optic lobes (Monsma and Booker, 1996a). Both developmental hormones and retinal afferents appear to regulate this process (Monsma and Booker, 1996b). The extent of PCD in other regions of the developing *Manduca* brain is largely unstudied. A general role for ingrowing sensory afferents in the regulation of neuronal survival is suggested by the sex differences that arise in populations of antennal lobe interneurons as a result of sexual dimorphisms in the antennae (Schneiderman *et al.*, 1982; Kalberer *et al.*, 2010). Recent studies attempting 3D reconstructions of the developing *Manduca* brain suggest a revival of interest in brain metamorphosis that may lead to new data on PCD in the brain (Huetteroth *et al.*, 2010). Several peptidergic neurons in the brain that might be expected to undergo PCD persist through the adult stage of life. These are the protocerebral prothoracicotrophic (PTTH) neurons, which regulate the synthesis and secretion of ecdysteroids by the prothoracic glands. PTTH activity and, presumably, PTTH neurons persist in the adult brain (Rybczynski *et al.*, 2009), despite the PCD of the prothoracic glands in the pharate adult (Dai and Gilbert, 1997).

12.4.4. Regulation of PCD in the Nervous System during Metamorphosis

The ecdysteroids couple neuronal PCD with other metamorphic changes. It is important to note that the ecdysteroid cue for triggering metamorphic neuronal death can be either a rising or a falling titer, depending upon developmental stage. For example, the decline in circulating levels of 20E that occurs at the end of adult development prior to adult eclosion is the cue for PCD of abdominal motoneurons at this time, and treatment with exogenous 20E blocks this PCD (Truman and Schwartz, 1984). By contrast, it is the pre-pupal rise in circulating ecdysteroids that is responsible for the larval–pupal transition death of the proleg motoneurons in abdominal ganglia A5 and A6 (Weeks and Ernst-Utzschneider, 1989; Weeks *et al.*, 1992). The response of the proleg motoneurons to the steroid signal, however, is segment-specific. Homologous neurons in abdominal ganglia A3 and A4 persist through the pupal stage and adult development, but then

undergo PCD within 24 hours of adult eclosion (Zee and Weeks, 2001). These responses to 20E, as well as the segment specificity of response at different stages in development, are retained when individual proleg motoneurons are cultured *in vitro*, providing evidence for the cell-autonomous, target-independent nature of these PCDs (Streichert *et al.*, 1997; Hoffman and Weeks, 1998).

While ecdysteroids regulate gene expression in the *Manduca* nervous system (see, for example, Garrison and Witten, 2010) the target genes that mediate neuronal PCD have not been identified. In *Drosophila*, the expression of the ecdysteroid receptor A (EcR-A) has been directly correlated with the occurrence of post-eclosion neuronal death in the CNS and transcriptional activation of the *reaper* and *hid* death genes (Robinow *et al.*, 1993; Jiang *et al.*, 2000). It is not known if a similar relationship prevails in *Manduca* neurons, although autoradiographic evidence has demonstrated that *Manduca* motoneurons fated to die at the start of adult life display nuclear concentration of radiolabeled ecdysteroids (Fahrbach and Truman, 1989).

Evidence that other signals fine-tune timing of the death of neurons during metamorphosis comes from several sources. Adult *Manduca sexta* emerge from their pupal cuticle in an underground pupation chamber, and then must dig to the surface before inflating their wings. Adult moths forced to continue digging for hours beyond the time this behavior would normally cease exhibited delayed death of abdominal motoneurons (Truman, 1983). In addition, transection of the ventral nerve cord prior to adult eclosion blocks the death of specific motoneurons in ganglia posterior to the point of transection, even in moths in which the levels of 20E undergo a normal decline (Fahrbach and Truman, 1987).

A well-studied example of a spared abdominal motoneuron is MN-12. Subsequent to ventral nerve cord transection, this supernumerary member of the adult abdominal ganglion maintains its normal central arborizations and electrophysiological properties, implying that the cell death program has been completely blocked in the absence of a descending signal (Fahrbach *et al.*, 1995; DeLorme and Mesce, 1999). Treatment of cultured abdominal ganglia with extracts prepared from ventral nerve cord restores the normal pattern of cell death to MN-12, but the active factor in the extracts remains to be identified (Choi and Fahrbach, 1995). Other examples of motoneuron death, such as the death of the accessory planta retractors (APRs) at the larval–pupal transition, however, are unaffected by cutting of the connectives prior to the normal time of death (Weeks and Davidson, 1994). This suggests that the phenomenon of interganglionic cell death signaling affects only a subset of neurons.

Because of the scattered and episodic nature of neuronal death during metamorphosis, and the unavailability of transgenic *Manduca* for analysis, little is known about the molecular mechanisms of neuronal PCD in this species.

Hormone-dependent neuronal PCD is blocked by treatment with inhibitors of transcription or translation (Weeks *et al.*, 1993; Fahrbach *et al.*, 1994; Ewer *et al.*, 1998; Hoffman and Weeks, 1998). In support of the hypothesis that PCD of *Manduca* neurons requires *de novo* protein synthesis, a two-dimensional gel electrophoresis analysis of the *Manduca* abdominal ganglia revealed changes in protein expression patterns associated with newly-eclosed adults, a period of massive PCD. These changes included expression of novel proteins (Montemayor *et al.*, 1990). In cultured proleg motoneurons, inhibition of caspase activity blocked PCD (Hoffman and Weeks, 2001), but ultrastructural studies of the APR motoneurons and the motoneurons that innervate the ISMs indicate that neuronal death in *Manduca* during metamorphosis may be autophagic rather than apoptotic, or combine features of both PCD programs (Stocker *et al.*, 1978; Kinch *et al.*, 2003).

Immunolabeling studies, in which the distribution of several death-associated gene products (initially identified from a screen of dying moth muscle; see section 12.5.4.3 and Table 1) was examined in the segmental ganglia, failed to reveal a reliable correlation of enhanced ubiquitination- or multicatalytic proteinase-immunoreactivity within dying neurons (Fahrbach and Schwartz, 1994; Hashimoto *et al.*, 1996), despite association of these gene products with PCD in insect skeletal muscles (Haas *et al.*,

1995; Jones *et al.*, 1995). This neuron–muscle discrepancy may reflect that the basal levels of these components are sufficient to mediate neuronal PCD, but are inadequate for the destruction of the giant muscle fibers. By contrast, apolipoprotein III is upregulated both by dying neurons and by degenerating muscles in *Manduca*, a finding that suggests that this molecule has functions in PCD in addition to its role in lipid transport (Sun *et al.*, 1995).

12.4.5. PCD of Muscles during Metamorphosis

The ISMs of *Manduca* (Figure 1) are the major abdominal muscles of the larva, pupa, and pharate adult. The ISMs are divided into separate pairs of bilaterally symmetric bundles, each of which attaches to the cuticle at the inter-segmental boundaries. These muscles form in the embryo, and span eight of the abdominal segments in the larva. The ISMs provide the propulsive force for hatching and subsequent larval locomotion. Following pupation, the muscles in the first two and last two abdominal segments die and rapidly disappear. The muscles in the middle four segments persist throughout metamorphosis, and are used for the defensive and respiratory movements of the pupa. Following adult eclosion, the remaining ISMs undergo PCD during the subsequent 30 hours. While the basis for this segmental fate determination has not been examined, presumably it is established early in embryogenesis as a

Table 1 Genes Differentially Expressed in Condemned *Manduca* ISMs

Process	Gene	Response	Reference
Proteolysis	Polyubiquitin	Induced	Schwartz <i>et al.</i> (1990b)
	14-kDa E2 ubiquitin conjugase	Induced	Haas <i>et al.</i> (1995)
	18–56 (Sug1) 26S proteasome ATPase	Induced	Sun <i>et al.</i> (1996)
	28.1-kDa subunit catalytic subunit of 20S proteasome	Repressed	Löw <i>et al.</i> (2000)
	S6 (TBP7, MS73) 26S proteasome ATPase	Induced	Jones <i>et al.</i> (1995)
	S6' (TBP1) 26S proteasome ATPase	Induced	Löw <i>et al.</i> (2000)
	S7 (MSS1) 26S proteasome ATPase	Induced	Löw <i>et al.</i> (2000)
	S10b (SUG2) 26S proteasome ATPase	Induced	Löw <i>et al.</i> (2000)
	E75B	Repressed	Löw <i>et al.</i> (2005)
	Acheron (putative RNA binding protein)	Induced	Valavanis <i>et al.</i> (2007)
Transcription	elF1A Translation-Initiation Factor	Induced	Löw <i>et al.</i> (2005)
Translation	Oskar (maternal effect protein)	Repressed	Zhang <i>et al.</i> (2007)
	Small Cytoplasmic Repeat Protein	Induced	Kuelzer <i>et al.</i> (1999)
Signal transduction	SCLP)		
	G coupled receptor GPR85	Induced	Zhang <i>et al.</i> (2007)
	Calmodulin-dependent calcineurin A1 subunit	Induced	Zhang <i>et al.</i> (2007)
	Death Associated LIM-Only Protein (DALP) (insect ortholog of Hic-5)	Induced	Hu <i>et al.</i> (1999)
Metabolism	Apolipoprotein III	Induced	Sun <i>et al.</i> (1995)
	Low MW lipoprotein PBMHPC-23	Induced	Zhang <i>et al.</i> (2007)
	Hydroxy acid oxidase 1	Induced	Zhang <i>et al.</i> (2007)
Contractile protein	Actin	Repressed	Schwartz <i>et al.</i> (1993b)
	Myosin heavy chain	Repressed	Schwartz <i>et al.</i> (1993b)
	Myosin light chain	Repressed	Zhang <i>et al.</i> (2007)
	Myosin Regulatory Light Chain isoforms 1 and 2	Repressed	Zhang <i>et al.</i> (2007)
	Calponin 1	Repressed	Zhang <i>et al.</i> (2007)
	Troponin 1	Repressed	Zhang <i>et al.</i> (2007)

result of the actions of segmentation and homeotic genes (Bejsovec and Wieschaus, 1993; DiNardo *et al.*, 1994; French, 2001; Sanson, 2001).

The nuclear changes that accompany ISM death display none of the features of apoptosis (Schwartz *et al.*, 1993a) (Figure 1). The chromatin does not become electron-dense, but remains dispersed throughout the nucleoplasm. In addition, agarose gel electrophoresis of ISM genomic DNA fails to reveal apoptotic ladders. Ultrastructurally, there is an increase in autophagic vesicles, and the death of these cells is accompanied by autophagy (Lockshin and Beaulaton, 1974, 1979).

Following PCD of muscles in many animals, the cell corpse is phagocytosed by neighboring cells or circulating macrophage-like cells (Hart *et al.*, 2008). A classic example of this phenomenon is found in amphibian metamorphosis, where the massive tail musculature is lost during the transition from larva to adult (Weber, 1964; Watanabe and Sasaki, 1974). During this process, muscle fibers become decorated with macrophages that contain identifiable remnants of skeletal muscle debris (Metchnikoff, 1892; Nishikawa *et al.*, 1998). While dying muscles in insects are sometimes phagocytosed by circulating hemocytes (Crossley, 1968), this is not universally so (Jones *et al.*, 1978). In particular, the death of the ISMs following adult eclosion in moths does not attract macrophage-like cells, or rely on phagocytosis for resolution (Beaulaton and Lockshin, 1977) (Figure 1). In fact, estimates of ISM volumes and hemocyte numbers in adult *Manduca* suggest that removal of dying cells in these animals would require at least an order of magnitude greater number of phagocytic cells than has been shown to reside in the hemolymph (Jones and Schwartz, 2001).

While the ISMs of adult moths are not phagocytosed, Rheuben (1992) observed an intimate association between phagocytic hemocytes and the sarcolemma during the death of mesothoracic muscles in pupae. The phagocytes were well spaced along the fibers, and appeared to degrade the basal lamina. One difference between the ISMs and the mesothoracic fibers is that the latter are not completely degraded during development. Instead, they act as scaffolds for myoblasts that remodel the fibers during formation of adult muscle fibers. Phagocytes may play a more significant role in tissue remodeling rather than cell death.

12.4.5.1. Endocrine control of ISM death Timing of ISM death must be coordinated with other metamorphic events, or the animal might suffer disastrous consequences. For example, premature loss of the ISMs in moths would leave the animal trapped within the pupal cuticle and locked in either a cocoon or an underground chamber. Delays in ISM death might have other deleterious consequences, including depriving the adult of nutrients required for gametogenesis. As described in section

12.4.4, the titer of ecdysteroids serves as an endogenous developmental time reference that can be used by the different organs of the pupa to coordinate developmental decisions, including the timing of ISM death.

Early reports suggested that the cessation of motoneuron activity was the proximal trigger for ISM death (Lockshin and Williams, 1965b). Subsequent studies demonstrated that the timing of ISM death in *Antheraea polyphemus* was not altered by silencing motoneuron activity with the sodium channel blocker tetrodotoxin or by removal of the entire ventral nerve cord (Schwartz and Truman, 1983, 1984a). Instead, in this species, the trigger for cell death is the peptide eclosion hormone (EH) (Schwartz and Truman, 1984a, 1984b). EH acts via cGMP; the description of its role in ISM death represented the first study demonstrating that cGMP met all of Earl Sutherland's requirements for identifying a second messenger for action of a hormone (Sutherland, 1972; Schwartz and Truman, 1984b). The capacity of EH to act on the ISMs is itself under the control of circulating ecdysteroids, as a decline in 20E regulates both the timing of EH release and the capacity of the ISMs to respond to this trigger (Truman, 1984; Schwartz and Truman, 1984a). The possible role of other insect peptides, such as ecdysis-triggering hormone (ETH), in PCD has not been explored.

12.4.5.2. Physiology of ISM death The size of the ISMs, and the coordinated nature of the developmental changes that take place in this tissue during metamorphosis, facilitate examination of the physiological changes that accompany naturally occurring muscle atrophy and PCD. Under laboratory conditions, metamorphosis in *Manduca* is complete in 18 days, with adult eclosion taking place late on day 18. On day 15 of adult development the mass of the ISMs begins to decline, and during the next 3 days ISMs lose 40% of their mass. This pre-eclosion program of atrophy is non-pathological, and the muscles retain almost all of their normal physiological responses, including force/cross-sectional area and sensitivity to calcium ions in skinned fiber preparations (Schwartz and Ruff, 2002). These observations suggest that the reduction in muscle mass observed during the atrophy phase reflects a generalized enhancement of protein turnover rather than selective destruction of contractile proteins, despite the fact that entire contractile bundles are lost during this phase (Lockshin and Beaulaton, 1979).

The ISMs of *Manduca* begin PCD coincident with adult eclosion. At this time, the ISMs begin to lose mass at a rate of approximately 4% per hour (Schwartz and Ruff, 2002). By 24 h post-eclosion, reliable resting potentials can no longer be recorded (Lockshin, 1973). While there are few changes in the organization of the contractile apparatus during the atrophy phase, the post-emergence period is marked by profound sarcomere disruption (Lockshin and Beaulaton, 1979). Whole filaments disappear rapidly, with

a preferential loss of thick filaments relative to thin filaments (Beaulaton and Lockshin, 1977). During this same period mitochondria are lost, autophagic vacuoles form, and the T tubule system swells. As a consequence, the muscle fibers rapidly weaken, even when force is normalized to cross-sectional area. This is true for twitches, and for tetanus and caffeine-induced contractions (Schwartz and Ruff, 2002). There are also defects in the ability of the contractile apparatus to respond to free calcium in both intact muscles and skinned fiber preparations.

12.4.5.3. Patterns of gene expression during PCD of ISMs The primary biochemical mechanism that mediates the atrophy phase appears to be an increase in the ubiquitin–proteasome pathway, which allows protein catabolism to outstrip synthesis (Haas *et al.*, 1995). There is a transient increase in polyubiquitin expression in the ISMs on days 15 and 16 of pupal–adult development during the early phases of atrophy that is controlled by the falling ecdysteroid titer (Schwartz *et al.*, 1990b).

Lockshin (1969) demonstrated that ISM PCD in silkworms is blocked by inhibitors of RNA or protein synthesis, suggesting a requirement for *de novo* gene expression. These results are similar to those reported for PCD in other tissues, including the insect nervous system, as well as in other metamorphosing taxa such as amphibians (Weber, 1965).

To identify genes that may mediate ISM death, Schwartz and colleagues utilized a differential screening approach using cDNA libraries constructed from Day 18 *Manduca* ISM mRNA (Schwartz *et al.*, 1990). Even though the ISMs are dying, the abundance of most transcripts was unchanged during the last days of pupation. However, a few transcripts were found that dramatically induced or repressed with the commitment of the cells to die. The cloning of these differentially expressed genes resulted in the first identification of death-associated gene expression from any organism (Schwartz *et al.*, 1990b). Among the genes that are repressed when the ISMs become committed to die are actin and myosin heavy chain (Schwartz *et al.*, 1993b) (Table 1; Figure 3). These transcripts are among the most abundant in the muscle during larval and pupal life, but begin to disappear late on day 17 (the day before adult eclosion), and are almost undetectable by late day 18 when the animals eclose.

One mechanism for reducing transcript abundance is transcriptional repression. A complementary mechanism is enhancement of transcript degradation. In this regard, it was found that there is a transient increase in endogenous ISM RNase activity on day 17, which facilitates removal of transcripts prior to induction of new gene expression (Cascone and Schwartz, 2001). Coordinated control of transcription and degradation may allow the muscles to rapidly shift developmental programs from homeostasis to death. While the molecular mechanism

that mediates this global change in transcript abundance has not been determined, microRNAs are potential regulators. MicroRNAs bind to sequences within the 3' untranslated region (UTR) of target mRNAs and regulate transcript stability and translatability (Fabian *et al.*, 2010). In the case of *Manduca*, the stability of ISM transcripts could be transferred to ectopic reporter mRNAs by swapping the 3' UTRs (Cascone and Schwartz, 2001). In other models, such as *Drosophila*, microRNAs can exert a profound effect on developmental processes (Jones and Newbury, 2010). Specific microRNAs, such as Let-7C, control the timing of intersegmental muscle cell death following adult eclosion (Sokol *et al.*, 2008).

A small number of induced cell death-associated genes were identified from the ISM screen (Schwartz *et al.*, 1990b). Some encoded known proteins, including polyubiquitin (Schwartz *et al.*, 1990b) and several proteasome subunits (Sun *et al.*, 1996), while others encoded novel proteins (Hu *et al.*, 1999; Kuelzer *et al.*, 1999; Valavanis *et al.*, 2007). More recently, additional PCD-associated genes expressed in ISMs have been identified either via

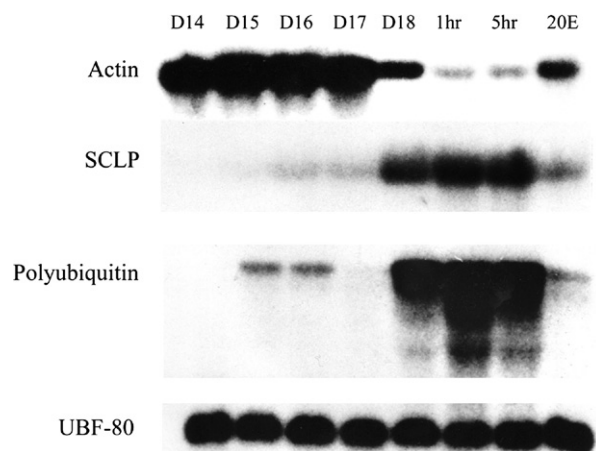


Figure 3 The ISMs begin to atrophy on day 15 of pupal–adult development and then initiate programmed cell death coincident with adult eclosion late on day 18. By 5 hours post-eclosion, the muscles have lost significant mass and physiological function. Most genes are constitutively expressed, like the ubiquitin fusion 80 (UBF-80) gene, which plays a role in ribosome biogenesis. While most genes are constitutively expressed within the ISMs, independent of developmental stage, a small number are induced or repressed with the commitment to die. Actin mRNA goes from being one of the most abundant transcripts within the ISMs prior to eclosion to all but disappearing in the dying cells. Conversely, Small Cytoplasmic Leucine Rich Repeat Protein (SCLP) is almost undetectable in the muscles prior to day 18 and is then dramatically induced with the commitment to die. Polyubiquitin is transiently induced on day 15, coincident with the onset of atrophy, and is then expressed at very high levels on day 18. All of the death-associated changes in transcript abundance can be prevented with injection of 25 µg of 20-hydroxyecdysone (20E) on day 17. D, day of pupal–adult development; h, hours post-eclosion; 20E, 20-hydroxyecdysone. Adapted from Schwartz (2008).

proteomics (Löw *et al.*, 2000, 2005; Zhang *et al.*, 2007) or by direct examination of proposed candidates in both *Manduca* and *Bombyx* (Table 1). The following section will focus on the genes identified from the molecular screen.

Ubiquitin is a 76 amino-acid peptide that is the most highly conserved protein present in all eukaryotes. At the protein level, insect and human ubiquitins are identical (Rechsteiner, 1988). The post-translational covalent attachment of ubiquitin to selected lysine residues on substrate proteins serves as a molecular tag to target proteins to specific fates within the cell (Salomons *et al.*, 2010). The addition of single ubiquitin moieties directs proteins to specific subcellular locations, while addition of multiple head-to-tail ubiquitin chains promotes binding to the 26S proteasome. This multisubunit protease then releases the ubiquitin, unfolds the substrate, and rapidly degrades it to small peptides.

As mentioned above, there is a transient increase in polyubiquitin expression that correlates with ISM atrophy. Polyubiquitin mRNA then accumulates to prodigious levels on day 18, along with the coordinated expression of both 20S and 26S proteasome subunits (Schwartz *et al.*, 1990b; Dawson *et al.*, 1995; Haas *et al.*, 1995; Jones *et al.*, 1995; Takayanagi *et al.*, 1996; Löw *et al.*, 1997). This enhancement in ubiquitin-dependent proteolysis is presumably adaptive, because the ISMs are not phagocytosed, and therefore require a cell-autonomous mechanism for the liberation of cellular constituents (Jones and Schwartz, 2001). The ubiquitin–proteasome pathway is presumably serving roles in both cell death signal transduction and large-scale protein turnover (Broemer and Meier, 2009). In *Manduca*, injection of day-18 pharate adults with proteasome inhibitors (hemin and N-acetyl-leu-leu-norleucinal) delayed ISM death (Bayline *et al.*, 2005).

Other known genes are induced in the dying ISMs, but their role in PCD is unknown. For example, the abundance of apolipoprotein III (apoLp-III) is dramatically induced, at both the RNA and protein levels, in both the ISMs and a subpopulation of neurons undergoing PCD (Sun *et al.*, 1995). ApoLp-III is synthesized predominantly in the fat body, and normally facilitates lipid transport in the hemolymph by associating with lipophorin. Given that the ISMs do not express lipophorin, the role of ApoLp-III in PCD is currently mysterious.

The majority of cDNAs isolated in the ISM screen encoded novel proteins of unknown function. An example is *SCLP* (small cytoplasmic leucine-rich repeat protein), which is induced at both the RNA and protein levels in condemned ISMs expressed on day 18 (Kuelzer *et al.*, 1999). This small protein is composed of multiple leucine-rich repeat protein–protein interaction motifs, and likely serves as a signal transduction protein. Ectopic expression of *SCLP* in different tissues in *Drosophila* did not result in an overt phenotype (Kuelzer *et al.*, 1999).

Two of the novel genes identified in the ISM screen do have vertebrate homologs. *DALP* (death-associated LIM-only protein) contains one perfect and two imperfect LIM domains (Hu *et al.*, 1999), structural motifs that consist of paired zinc fingers that mediate protein–protein interaction (Rétaux and Bachy, 2002). *DALP* is induced on day 17, well in advance of the other death-associated cDNAs from *Manduca* ISMs. Expression of the *DALP* protein is likely restricted to the ISMs, as it was not detected in flight muscle, fat body, Malpighian tubules, the ovary, oocytes, or the male sexual accessory gland. As with *SCLP*, the function of *DALP* was explored using transgenic flies (Hu *et al.*, 1999). Ectopic expression of *Manduca* *DALP* in the abdominal ISMs of fly pupae resulted in the disorganization of the contractile apparatus and subsequent muscle atrophy. Targeted mutations in the LIM domain blocked muscle atrophy, suggesting that the observed effects of ectopic *DALP* expression were dependent on expression of the intact functional protein.

Further insights into the function of *DALP* were gained by examining the effects of expressing *Manduca* *DALP* in the mouse myoblast C₂C₁₂ line (Hu *et al.*, 1999). This muscle satellite cell line has been extensively used as a model for examining muscle differentiation and PCD in mammals (Schwartz *et al.*, 2009). C₂C₁₂ cells can be maintained as a stable, non-transformed line that, when incubated in a low serum medium, ceases cycling and differentiates into multinucleated myotubes (Yaffe and Saxel, 1977). Expression of *DALP* blocked the differentiation of C₂C₁₂ cells into myotubes by blocking induction of MyoD, a basic helix–loop–helix muscle transcription factor required for differentiation. Effects of *DALP* were overcome by co-transfecting cells with an expression vector driving production of MyoD. In addition to blocking differentiation, *DALP* enhanced the probability of cell death. Identical results were obtained with C₂C₁₂ cells transformed to express *Hic-5* (hydrogen peroxide-inducible clone-5), the mammalian ortholog of *Manduca* *DALP*. These data show that *DALP* and *Hic-5* are likely conserved proteins that function as negative regulators of muscle differentiation and survival in insect and mammalian cells.

Another of the novel cell death associated genes from *Manduca* may play a role in human pathogenesis. Acheron (Achn) contains three Lupus antigen (La) repeats, nuclear localization and export (NLS and NES) signals, and an RNA recognition motif (Valavanis *et al.*, 2007). In fact, Achn defines a new subfamily of Lupus antigen (La) proteins that appears to have branched from authentic La protein relatively late in metazoan evolution. In mammalian cells, Achn (also known as La related protein 6, or LARP6), binds to the 5′ untranslated region of collagen mRNA and facilitates translation (Cai *et al.*, 2010). While its role in ISM death has not been explored, Achn plays an essential role in myogenesis in zebrafish (Wang *et al.*, 2009).

In C₂C₁₂ myoblasts, Achn acts upstream of MyoD and is required for these cells to either differentiate or undergo apoptosis following loss of growth factors (Wang *et al.*, 2009). Other studies have explored the role of Achn in regulating integrin–extracellular matrix interactions required for myogenesis. Both control C₂C₁₂ myoblasts and those engineered to express ectopic Achn expressed the fibronectin receptor integrin $\alpha 5 \beta 1$ in the presence of growth factors and the laminin receptor $\alpha 7 \beta 1$ following growth factor withdrawal. Expression of the laminin receptor was blocked in cells expressing either Achn antisense or dominant-negative Achn. Control cells and those expressing ectopic Achn undergo sequential and transient increases in both substrate adhesion and migration before cell fusion. Blockade of Achn expression reduced these effects on laminin but not on fibronectin. Taken together, these data suggest that Achn may influence differentiation in part via its control of cell adhesion dynamics (Glenn *et al.*, 2010).

A recent study has demonstrated that Achn is expressed in the myoepithelial cells of the mammary gland (Shao *et al.*, 2011). Microarray and immunohistochemical analysis of tissues from patients with breast cancer have demonstrated that Achn expression is significantly elevated in some basal-like tumors, the most aggressive of the breast cancers. Ectopic expression of Achn in MDA-MB-231 breast cancer cells induced a number of phenotypic changes that are associated with malignancy and metastasis, including enhanced cell proliferation, lamellipodia formation, greater invasive activity, and elevated expression of the metastasis-associated proteins MMP-9 and VEGF. In xenograph studies using athymic mice, MDA-MB-231 cells expressing ectopic Achn displayed enhanced angiogenesis and an approximately five-fold increase in tumor size relative to control cells. These data support the hypothesis that Achn enhances human breast tumor growth and vascularization, and may represent a target for diagnostics and therapeutics.

12.5. The *Drosophila* Model

12.5.1. Choice of *Drosophila melanogaster* as a Model System for the Study of PCD of Nerve and Muscle Cells

Like moths, fruit flies are holometabolous insects. Three larval (feeding) stages are followed by pupation. The pupa forms inside the pupal case (puparium), the hardened cuticle of the final larval stage. *Drosophila* development is rapid and the entire life cycle is complete within 10 days at 25°C. While *Drosophila* have provided a powerful genetic model system for a century, it arrived relatively late to the modern study of PCD. Thus, it was not until 1990 that Kimura and Truman (1990) extended classic observations of muscle death during *Drosophila* metamorphosis

by systematically documenting neuronal death in the fused ventral ganglia following adult eclosion. Injection of living flies with a toluidine blue solution revealed numerous examples of dying neurons in the dorsal and lateral regions of the abdominal and metathoracic neuromeres. These investigators also used bi-refringence to map degeneration of head and abdominal musculature during metamorphosis and adult eclosion. Several studies described below have made use of these baseline observations to explore ecdysteroid regulation and molecular mechanisms of PCD in the nervous system of newly-eclosed adult flies (Robinow *et al.*, 1993, 1997; Draizen *et al.*, 1999).

Interestingly, the first insight into the genetic basis of PCD in flies did not come from studies of metamorphosis but, rather, of embryogenesis. The laboratory of Hermann Steller used a classic histological technique, acridine orange staining, to identify dying cells with an apoptotic phenotype in wild type embryos (Abrams *et al.*, 1993). A subsequent genetic screen using a set of 129 chromosomal deficiency strains identified a small region at 75C1,2 on chromosome 3L that is essential for developmental and X-irradiation induced apoptosis (White *et al.*, 1994). Subsequent studies led to the identification of four related pro-apoptotic genes that reside within a 300-kb interval in this region: *reaper* (*rpr*), *head involution defective* (*hid*), *grim*, and *sickle* (*skl*) (White *et al.*, 1994; Grether *et al.*, 1995; Chen *et al.*, 1996; Christich *et al.*, 2002; Srinivasula *et al.*, 2002; Wing *et al.*, 2002) (Figure 4). A genetic modifier screen resulted in the identification of *Drosophila* Inhibitor of Apoptosis Proteins, DIAP1 and DIAP2, which are crucial inhibitors of caspase activities (Hay *et al.*, 1995). DIAP1 mutants exhibit embryonic lethality associated with massive ectopic PCD, indicating it is essential for survival of many cell types (Hay, 2000).

The Reaper, Grim, and Sickle proteins are small (65, 138, and 108 amino acids, respectively), while Hid is substantially larger (410 amino acids). Each possesses a related 14-aa region at the N-terminus, designated the RHG (Reaper, Hid, Grim) motif or IBM (inhibitor of apoptosis (IAP)-binding motif). This motif has potent pro-apoptotic activities, and can bind to and repress the caspase-inhibiting activities of IAPs (Vaux and Silke, 2005; Steller, 2008; Orme and Meier, 2009; also discussed below). The RHG/IBM motifs of Reaper and Grim share 71% identity, with three of the four amino acid differences conservative substitutions. However, despite this strong similarity, *in vivo* studies have shown that these domains possess distinct death-inducing activities (Wing *et al.*, 1998). More recently, two unlinked *Drosophila* genes, *omi/htr2A* (a homolog of the mammalian Htr2A serine protease) and *jafrac2* (a thiodoxin peroxidase), were identified that encode proteins with similar RHG domains (see, for example, Challa *et al.*, 2007), but for this review we will focus on the four linked RHG genes, *reaper*, *hid*, *grim*, and *sickle*. Reaper, Grim, and

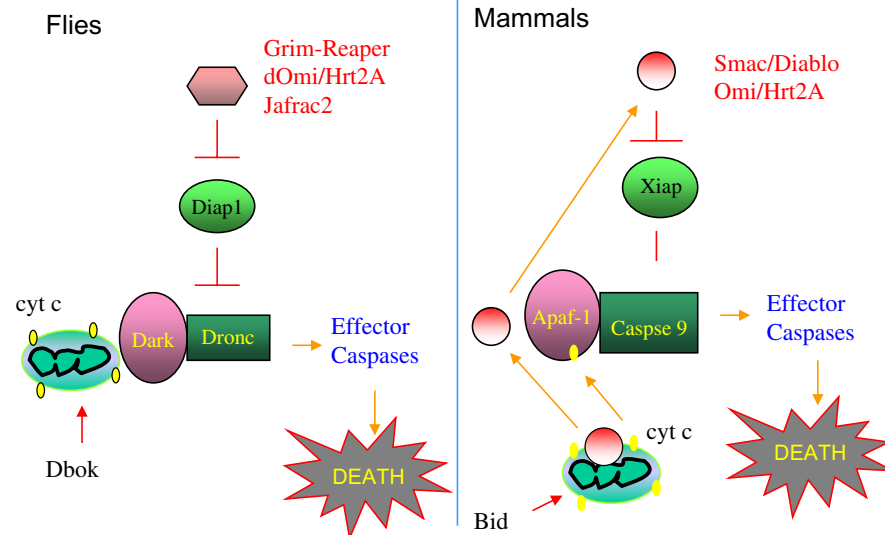


Figure 4 The *grim-reaper* genes encode related pro-apoptotic proteins. (A) The *hid*, *grim*, *reaper*, and *sickle* genes all reside within a 300-kb interval in the 75C region of chromosome 3L of *Drosophila melanogaster*. The four genes are transcribed in the same direction (arrows above genes) and are expressed predominantly in doomed and dying cells. No genes are predicted to reside between *grim* and *reaper*, or *reaper* and *sickle*, while four unrelated genes (orange blocks) reside between *hid* and *grim*. (B) *reaper*, *sickle*, and *grim* encode small proteins that contain an N-terminal RHG motif/IBM as well as a C-terminal Trp block/GH3 domain. Hid is a substantially larger protein that also contains an RHG motif but does not exhibit strong similarity to the Trp block/GH3 domain.

Sickle also share a second region of sequence similarity, a 15-aa Grim helix 1, 2, and 3 (GH3) domain or Trp block (Figure 4), which has distinct pro-apoptotic functions. This domain is necessary for mitochondrial association of Reaper and Grim during apoptosis, and activates PCD independently of the IAP antagonizing functions of the NH2 RHG domain/IBM (see, for example, Abdelwahid *et al.*, 2007). Hid possesses four regions that resemble a Trp block/GH3 domain, although the level of similarity is much lower (Wing *et al.*, 2001). In addition, Hid also contains a mitochondrial localization signal and can form a heterodimer with Reaper that translocates to mitochondria to promote cell death (Sandu *et al.*, 2010). Reaper also forms homodimers and heterodimers with Grim but not Sickle. Formation of these dimers appears essential for strong pro-apoptotic activity of RHG proteins (Sandu *et al.*, 2010). Finally, Reaper and Grim, but not Hid, can induce apoptosis via general translation inhibition (Holley *et al.*, 2002; Yoo *et al.*, 2002). The related RHG proteins are multifunctional apoptosis activators that have distinct and overlapping modes of action.

Insights into the molecular mechanisms of PCD gained by further studies of RHG genes during *Drosophila* embryonic development and metamorphosis are described in the following sections. Significantly, targeted ectopic expression of these genes in cells that are normally fated to live has provided an invaluable tool for producing cell-specific lesions of the fly CNS, particularly of peptidergic neurons (see, for example, McNabb *et al.*, 1997;

Zhou *et al.*, 1997; Renn *et al.*, 1999; Rulifson *et al.*, 2002; Park *et al.*, 2003; Zhao *et al.*, 2010).

12.5.2. Hormonal Regulation of Metamorphosis in *Drosophila melanogaster*

Hormonal regulation of metamorphosis in *Drosophila* is similar to that described for other insects, with post-embryonic development wholly dependent upon exposure of tissues to coordinated pulses of 20E, which in turn produce a coordinated cascade of gene expression (Riddiford, 1993; Truman and Riddiford, 2002). Cloning of the *Drosophila* ecdysone receptor (*EcR*) gene led to studies of the expression of *EcR* in tissues including neurons and muscle (Koelle *et al.*, 1991; Robinow *et al.*, 1993; Talbot *et al.*, 1993; Truman *et al.*, 1994). These studies support the view, based on earlier studies of *Manduca*, that ecdysteroid regulation of PCD in insects is a result of direct action of the steroid on the cells that are fated to die.

12.5.3. PCD of Neurons during Early Development and Metamorphosis

Four periods of PCD have been described in the nervous system of *Drosophila*: during mid-to-late embryogenesis, in late third instar larvae, in pupae during metamorphosis, and in the newly-eclosed adult. The PCD that occurs during these stages generates in turn the larval and adult nervous systems by eliminating cells that are produced in excess, such as the embryonic midline glia, and cells with

transient functions, such as larval abdominal ganglion neuroblasts (Kimura and Truman, 1990; Truman *et al.*, 1993; Sonnenfeld and Jacobs, 1995; Zhou *et al.*, 1995). As in mammalian development, the extent of cell death in the embryonic *Drosophila* nervous system is profound; approximately two-thirds of all cells born die before larval hatching (Abrams *et al.*, 1993; White *et al.*, 1994).

The extent of cell death in *Drosophila* embryos suggests that this process is critical for development of the larval nervous system. However, homozygous Df(3L)H99 mutant embryos that lack cell death exhibit relatively normal organization of the ventral nerve cord despite having a large excess of surviving cells (White *et al.*, 1994; Zhou *et al.*, 1995). Nonetheless, mutations in several genes that are important for PCD result in hypertrophy of the embryonic, larval, or adult CNS, and these mutants often exhibit stage-specific lethality or sterility (Grether *et al.*, 1995; Song *et al.*, 1997; Peterson *et al.*, 2002; Rogulja-Ortmann *et al.*, 2007; Kumar *et al.*, 2009). Removal of dead cells from the CNS also appears to be important for nervous system development, as mutants lacking functional macrophages exhibit disruptions in the normal architecture of the embryonic axon scaffold (Sears *et al.*, 2003).

The proportion of neurons that die during the post-embryonic period is lower than during embryogenesis, and the widespread degeneration of larval tissues during metamorphosis in flies does not extend to the CNS. As in *Manduca*, most neurons in the larva persist rather than die (Truman, 1990). After pupariation, however, many dying neurons can be observed in the ventral CNS in both thoracic and abdominal neuromeres (Truman *et al.*, 1993). The identity of most of these neurons has not been determined, with the exception of several motoneurons identified by retrograde fills of the T2 mesothoracic nerve (Consoulas *et al.*, 2002). In addition, PCD of neuroblasts after pupariation marks the termination of post-embryonic neurogenesis in the ventral nervous system (Truman and Bate, 1988; Truman *et al.*, 1993; Kumar *et al.*, 2009). As in *Manduca*, identified peptidergic neurons provide a population that can be tracked across metamorphic transitions. Corazonin is an 11-amino acid peptide expressed by 8 pairs of neurons in the ventral nerve cord of larval fruit flies. Detailed studies of the time-course of corazonin disappearance revealed that these neurons undergo PCD 2–6 hours after puparium formation. Because their fate can be readily tracked by anti-corazonin immunolabeling, they were chosen to explore the ecdysteroid-dependent and cell-specific mechanisms of PCD (Choi *et al.*, 2006). These studies revealed that the pulses of ecdysteroids that drive the onset of metamorphosis trigger the death of the corazonin neurons via activation of the EcR nuclear receptor. The *EcR* gene of *Drosophila* encodes three ecdysone receptor subunits: EcR-A, EcR-B1, and EcR-B2 (Talbot *et al.*, 1993). These receptor subunits share common DNA and ligand-binding domains, but have different

N-terminal regions. Analysis of loss-of-function mutants for specific EcR isoforms revealed that either EcR-B1 or EcR-B2 can activate the cascade of transcriptional events that results in the PCD of these neurons (Choi *et al.*, 2006).

The use of EcR isoform-specific antibodies also revealed that a high level of expression of EcR-A is correlated with post-eclosion PCD (Robinow *et al.*, 1993). Prior to eclosion, nearly 300 neurons in the ventral ganglia displayed high levels of EcR-A immunoreactivity. During the first 24 hours after adult eclosion, essentially the EcR-A-immunopositive neurons in the ventral ganglia displayed characteristic features of PCD and were basically all absent by the end of this first day. In contrast to the death of the corazonin neurons at the end of larval life described above (Choi *et al.*, 2006), it appears that it is EcR-A expression during the period of ecdysteroid decline at the end of metamorphosis which is critical to the decision of these neurons to commit suicide. A subsequent analysis of EcR-A mutants supported the view that the distinct EcR-isoforms have specific functions during development, and linked EcR-A receptors to PCD in an additional tissue, the larval salivary glands (Davis *et al.*, 2005). Analysis of isoform-specific activities of EcR is an active area of research with a current focus on the regulatory role of heterodimerization partners (Braun *et al.*, 2009).

12.5.4. Molecular Mechanisms of Neuronal Death

PCD research in *Drosophila* is a large and active field that can no longer be adequately summarized in a single chapter. We focus here on selected topics related to the death of neurons and glial cells, covering both apoptosis and autophagy. Accounts of the control of neuron number by neuroblast apoptosis in the ventral nerve cord and the brain can be found in Peterson *et al.* (2002), Bello *et al.* (2003, 2007), Kumar *et al.* (2009), and Siegrist *et al.* (2010). Descriptions of the regulation of cell survival by cell intrinsic mechanisms in sensory neuron lineages can be found in Spana and Doe (1996) and Orgogozo *et al.* (2002). These studies have provided insights into the regulation of apoptosis and other cell fates by asymmetric cell division (Hatzold and Conradt, 2008; Zhong, 2008).

12.5.4.1. The *Drosophila* cell death “machinery” for apoptosis The basic molecular machinery of apoptosis includes both initiator and executioner caspases, a Ced (cell death abnormal)-4/Apaf (apoptosome associated factor)-1 ortholog, and Ced-9/Bcl-2 protein family members (Tittel and Steller, 2000; Vernooy *et al.*, 2000) (Table 2). These proteins all have critical functions in apoptosis that were initially defined by studies in the nematode *Caenorhabditis elegans* (Horvitz *et al.*, 1994; Liu and Hengartner, 1999). Caspases are considered the

Table 2 *Drosophila* Cell Death Regulators

Family	Proteins
Caspases	Dronc
	Dcp-1
	Drice
	Dredd
	Strica/Dream
	Decay
	Damm
Caspase inhibitor	DIAP1/Thread
	DIAP2
Bcl-2 family	Drob-1/Debcl/Dborg-1/Dbok
	Buffy/Dborg-2
IAP inhibitors	Reaper
	Hid
	Grim
	Sickle
	Jafrac2
Apaf-1/Ded 4 family	Dark/HAC-1/Dapaf-1

cellular executioners because they activate proteolytic enzymes that digest cellular structural elements; Apaf-1 factors form complexes called apoptosomes that regulate caspase activity, and Bcl-2 family proteins regulate the integrity of mitochondrial membranes. An important difference between apoptosis in *C. elegans*, *Drosophila*, and mammals is the role of mitochondria (and, hence, an important role for Bcl-2 family members). In mammals, mitochondria undergo membrane permeability changes in dying cells and serve as critical sources of several pro-apoptotic factors, including cytochrome *c* and Smac/Diablo. However, while mitochondria have clearly been implicated in apoptotic pathways in flies and worms, the importance and extent of their contribution is less certain (see, for example, Colin *et al.*, 2009; Krieser and White, 2009). In flies, the major arbiter and point of regulation for cell survival decisions appears to be DIAP1 (Hay, 2000; Vaux and Silke, 2005; Orme and Meier, 2009).

In flies, worms, and mammals, caspases serve as the effectors of PCD. These specialized cysteine proteases cleave at aspartate or glutamate residues within enzymatic or structural substrate proteins to promote the dismantling of a cell (Cooper *et al.*, 2009; Feinstein-Rotkopf and Arama, 2009). Caspases are initially synthesized as inactive zymogens that contain either a long or a short prodomain, and a large and small subunit. Proteolytic cleavage of caspases in dying cells results in formation of an active heterotetramer comprised of two large and two small subunits. Heterotetramers derived from long prodomain zymogens correspond to initiator caspases, and these cleave short prodomain zymogens to generate active effector caspases that ultimately dismantle diverse cellular substrates. Thus, a cascade of caspase activities defines apoptotic cell deaths. The *Drosophila* genome encodes seven caspases (Table 2), including three long prodomain initiator caspases such as Dronc (*Drosophila* nedd2-like caspase,

similar to caspase 9), and four short prodomain effector caspases such as Drice (similar to caspase-3) and DCP-1 (death caspase 1; similar to caspase-7). Dronc and Drice are now recognized as key elements of the core of the *Drosophila* caspase-dependent cell death machinery (Hay and Guo, 2006). Mutant *Drosophila* embryos lacking zygotic *dronc* gene product display significantly reduced levels of apoptosis in many cell populations, including the developing ventral nerve cord (Chew *et al.*, 2004). During metamorphosis, targeted expression of the baculovirus pan-caspase inhibitor p35 blocks the death of the larval corazonin neurons of the ventral nerve cord (Choi *et al.*, 2006). Interestingly, a recent report links the regulation of Dronc activity (via phosphorylation at S130) to cellular metabolism by demonstration that increases in NADPH inhibit this caspase while inhibition of NADPH production triggers apoptosis (Yang *et al.*, 2010); this relationship between NADPH and Dronc activity was first demonstrated in *Drosophila* S2 cells, but was also found in neurons in the developing CNS. Focal activation of caspases plays a role in neurite pruning in *Drosophila*, which provides a surprising and elegant tool for sculpting the nervous system and refining synaptic connections (Williams *et al.*, 2006).

The fly genome encodes two Bcl-2 family members, Drob-1/Debcl/dBorg-1/dBok and Buffy/dBorg-2, that possess the Bcl-2 homology (BH) domains BH1, BH2, and BH3 (Brachmann *et al.*, 2000; Colussi *et al.*, 2000; Zhang *et al.*, 2000; Quinn *et al.*, 2003). Both of these proteins resemble the pro-apoptotic mammalian Bok protein, and act to promote apoptosis (Igaki and Miura, 2004). This difference supports the view that flies are less dependent upon mitochondrial disruption for the activation of caspases than are mammals (Wang and Youle, 2009). This observation provokes speculation concerning the evolution of cell death mechanisms, and raises the question: what factors inhibit death in insect cells?

12.5.4.2. Role of IAP and RHG family proteins in apoptosis

The major anti-apoptotic proteins in *Drosophila* are the IAPs (inhibitors of apoptosis proteins), which function by binding to and inactivating caspases (Hay, 2000; Vaux and Silke, 2005; Steller, 2008; Orme and Meier, 2009). IAPs were first identified from the *Cydia pomonella* granulosis virus, and found to inhibit apoptosis in infected host cells (Crook *et al.*, 1993). Subsequently, a large number of viral and cellular IAPs have been identified in divergent species that function as potent caspase repressors and inhibitors of PCD. IAPs all contain one or more copies of a 70-aa baculovirus IAP repeat (BIR) domain, typically located in the central or N-terminal region of the protein. In addition, they contain a 50-aa RING (Really Interesting New Gene) domain, generally situated towards the C-terminal region of the protein. The sequences of the BIR and RING

domains both resemble zinc fingers, and serve as critical protein–protein interaction domains. The BIR domains can directly associate with procaspases and caspases, and inhibit their activation or activities (Vaux and Silke, 2005; Steller, 2008; Orme and Meier, 2009). The RING domain possesses ubiquitin E3 ligase activity that can target bound caspases for polyubiquitination and degradation via the 26S proteasome (reviewed by Bergmann, 2010). In addition, IAPs bound to caspases are themselves targeted to proteasome-dependent proteolysis via the N-end rule pathway of protein turnover (Ditzel *et al.*, 2003). The IAPs therefore can efficiently reduce cellular caspase levels, and the interplay between caspases and IAPs is a key determinant of cell survival. The levels of IAPs themselves are also under rigid regulation. In particular, the RHG proteins bind not only to the BIR domains of IAPs and displace bound caspases, but also promote IAP auto-ubiquitination (Yang *et al.*, 2000; Yoo *et al.*, 2002). The release and de-repression of bound caspases as well as increased turnover of IAPs act together to strongly promote PCD.

The dependence on IAP proteins for cellular survival provides a key regulatory point for the regulation of apoptosis. The BIR domains of DIAP1 bind both caspases and the Grim-Reaper proteins. In particular, the BIR2 domain of DIAP1 associates both with Dronc and Reaper, Hid, Grim, and Sickie. The BIR1 domain binds to Drice and DCP-1 and also to Reaper and Grim, and, to some extent, Hid. Thus, the two BIR domains exhibit distinct abilities to bind to both specific caspases and RHG proteins (see, for example, Zachariou *et al.*, 2003). In surviving cells, DIAP1 binds and inhibits caspases, thereby repressing cell death (Figure 5). In contrast, in cells that receive

signals to die, Grim-Reaper proteins are expressed and compete with caspases for DIAP1 binding. The displacement of caspases from DIAP1 results in increased levels of proteolytically active enzymes that promote cell death. Cell survival decisions, therefore, are determined by the interactions among the pro-apoptotic RHG proteins, the anti-apoptotic IAPs, and caspases.

IAP regulation is a conserved mechanism for controlling cell survival (Figure 5). In mammals, Smac/Diablo and Omi/Htr2A are mitochondrial proteins released with cytochrome *c* in dying cells (Du *et al.*, 2000; Suzuki, 2001; Verhagen and Vaux, 2002). Cytoplasmic Smac/Diablo and Omi/Htr2A associates with X-linked inhibitor of apoptosis (XIAP) to prevent it from binding and thereby inhibiting caspase-9. Strikingly, the binding of Smac/Diablo and Omi/Htr2A to IAPs is mediated through an N-terminal tetrapeptide sequence that is conserved in the Grim-Reaper RHG motif, implying conserved modes of action for the fly and mammalian proteins (Chai *et al.*, 2000; Srinivasula *et al.*, 2000, 2002). As expression of IAPs is upregulated in many types of tumors, interest is intense regarding the possibility that either these endogenous inhibitors or related synthetic compounds can be exploited to develop new therapies for cancer and other diseases (Fulda, 2007; Gyrd-Hansen and Meier, 2010).

12.5.4.3. Regulation of embryonic glial cell survival in *Drosophila* One important mechanism for regulating the survival of neurons and glia within the vertebrate nervous system involves the actions of trophic factors (Raff *et al.*, 1993). These pro-survival molecules are synthesized in restricted amounts by target tissues, and permit winnowing of innervating cells to ensure matching

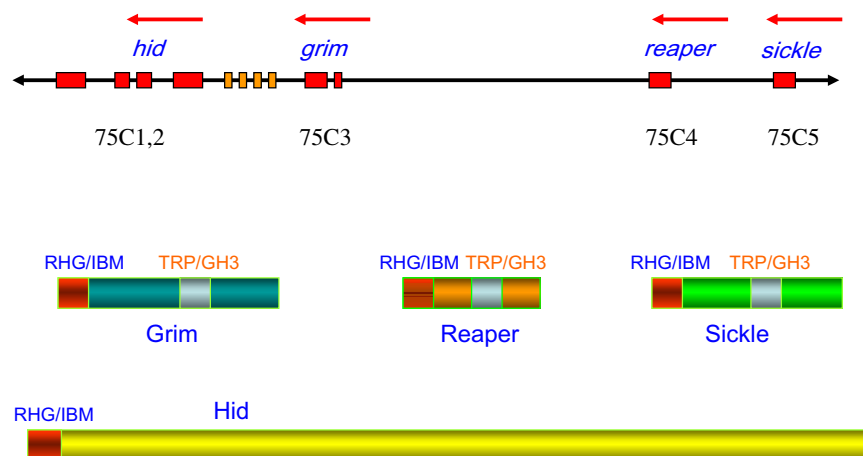


Figure 5 IAP inhibition is a conserved mechanism of regulation of apoptosis. In *Drosophila*, expression of the *grim-reaper*, *jafrac2*, or *dOmi/Htr2A* genes is activated in doomed cells, and the corresponding proteins bind to DIAP1 and block its ability to inhibit caspases. This results in active caspases that degrade cellular proteins to mediate apoptosis. In mammals, pro-apoptotic stimuli induce the release of Smac/Diabl, Omi/Htr2A, as well as other pro-apoptotic factors, including cytochrome *c*, from the mitochondria. Cytoplasmic Smac/Diablo or Omi/Htr2A binds XIAP and represses its caspase-inhibitory actions, thereby promoting apoptosis. In both flies and mammals, the orthologous Dark and Apaf-1 proteins as well as pro-apoptotic members of the Ced-9/Bcl-2 family promote activation of inhibitor caspases.

of interacting cell populations. Such molecules appear to be involved in regulating glial cell survival in *Drosophila* embryos.

Approximately 10% of all cells within the *Drosophila* nervous system are glial cells. Neurons and glia display complex signaling relationships during development. For example, the PCD of neurons de-represses division of glial cells, leading to glial cell proliferation (Kato *et al.*, 2009). Among the best-characterized glial cells are the embryonic longitudinal and midline glia of the CNS. In both these lineages, glial cells undergo extensive apoptosis during embryogenesis (Sonnenfeld and Jacobs, 1995; Zhou *et al.*, 1995; Kinrade *et al.*, 2001). The survival of subsets of these cell populations is governed by trophic actions of EGF-related ligands and activation of the Ras/MAP kinase pathway via the EGF receptor homolog (EGFR).

During embryogenesis, a single, laterally positioned glioblast precursor gives rise to approximately 10 longitudinal glia in each hemisegment of the ventral nerve cord. These glial cells migrate medially, contact pioneer longitudinal axons, and ultimately ensheath the longitudinal nerve bundles (Hidalgo and Booth, 2000). Coincident with the onset of axon/glial contact, many of these longitudinal glia undergo apoptosis, suggesting that as the glia contact the axons, they become dependent upon them for survival (Kinrade *et al.*, 2001). Consistent with this notion, ablation of pioneer and other neurons results in a decrease in longitudinal glia (Hidalgo *et al.*, 2001). Thus, apoptosis determines the final numbers of longitudinal glia during embryogenesis, and axon-derived factors are required for longitudinal glial cell survival. One of these factors is Vein (Vn), a *Drosophila* neuregulin homolog that contains both an IgG domain and an EGF domain (Schnepp *et al.*, 1996). The *vein* gene is expressed in a subset of neurons within the embryonic CNS including the midline precursor 2 (MP2) pioneer neurons and the Ventral Unpaired Median (VUM) neurons of the CNS midline (Hidalgo *et al.*, 2001). *Vein* mutants exhibit ectopic apoptosis of longitudinal glial cells, which is also observed for RNAi-mediated knockdown of *vein* gene product in either all neurons or the MP2 neurons. Vein is a secreted ligand for *Drosophila* EGFR, and EGFR-mediated activation of the Ras/MAP kinase pathway is essential for longitudinal glial cell survival. EGFR is transiently expressed in a subset of these glia, suggesting that Vein is secreted from axons to promote survival of these glial cells.

Similar to the Vein-dependent longitudinal glia, survival of the midline glia is also promoted by an EGF family member, the TGF- α homolog Spitz (Bergmann *et al.*, 2002). Midline glia are essential for proper formation of the axon scaffold, as migrating midline glia contact, separate, and ultimately ensheath the anterior and posterior axon commissures (Klambt *et al.*, 1991). The midline glia are normally in close contact with commissural axons, suggesting that glial-axon contact may be important for

midline glial cell survival. Observations made in *commissureless* mutants, where the commissures fail to form, are consistent with this notion; in these mutants, the isolated midline glial cells that fail to contact axons undergo apoptosis (Sonnenfeld and Jacobs, 1995). Ultrastructural and genetic analyses indicate that the midline glia undergo apoptosis, which reduces their number from an initial set of nine cells to three cells in each segment of the mature ventral nerve cord (Sonnenfeld and Jacobs, 1995; Zhou *et al.*, 1995). This apoptosis is dependent upon caspases and the actions of multiple Grim-Reaper proteins. Simultaneous loss of *reaper*, *hid*, and *grim* gene expression blocks all midline glial cell death, and results in the survival of the nine midline glia per segment. The loss of *hid* and *grim* results in approximately seven to eight midline glia per segment, while the loss of *hid* alone results in six midline glia (Zhou *et al.*, 1997; Bergmann *et al.*, 2002). Thus, *hid* expression is required for the death of three midline glia, and *reaper* and *grim* are together essential for the death of the other three midline glia. Both *hid* gene transcription and activity of Hid protein in the midline glia are regulated by proteins in the EGF signaling pathway, including Spitz and EGFR (Kurada and White, 1998). The loss of midline glia in *spitz* mutants is rescued in *spitz;hid* double mutants, implying that the pro-apoptotic functions of Hid are normally opposed by the prosurvival functions of Spitz.

The Spitz protein appears to be an axon-derived trophic factor required for midline glial survival (Bergmann *et al.*, 2002), as the loss of midline glia in *spitz* mutants is rescued by targeted expression of the transmembrane Spitz precursor protein in commissural neurons, and the number of surviving midline glia can be modulated by controlling the levels of Spitz activity. The model that has emerged is that axon-derived Spitz protein signals the midline glia via EGFR, resulting in activation of the Ras/MAP kinase pathway. Hid activity is subsequently downregulated via phosphorylation, which permits midline glial survival. Taken together, the actions of Vein in the longitudinal glia and Spitz in the midline glia indicate that trophic survival mechanisms are utilized to match the sizes of interacting populations of neurons and glial cells in invertebrates as well as vertebrates.

12.5.4.4. The *Drosophila* cell death “machinery” for autophagy Studies in *Drosophila* are now contributing significantly to our understanding of the molecular mechanisms of autophagy (Chang and Neufeld, 2010; Ryoo and Baehrecke, 2010; Figure 6). In animal cells, autophagy is regulated by the class I and class III phosphatidylinositol 3-kinase pathways (PI3K). Metabolic signals such as the binding of insulin to its receptor activate PI3K, which ultimately releases an autophagy inhibitor, TOR (target of rapamycin), from inhibition. The activation of TOR in turn activates

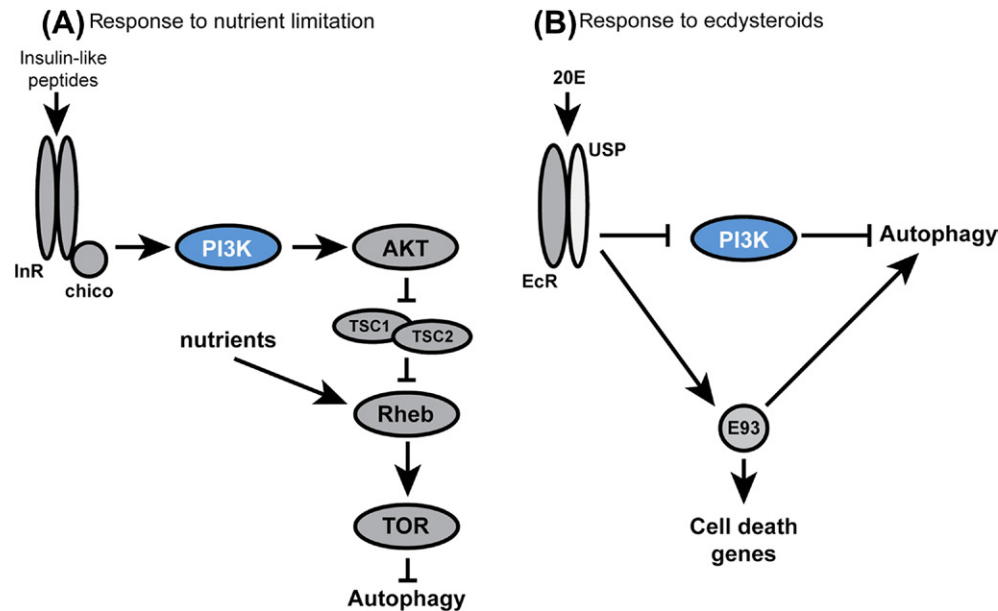


Figure 6 Simplified schematic overviews of established pathways for induction of autophagy in *Drosophila*. Other pathways have also been described. (A) Autophagy can be induced by nutrient limitation through inhibition of TOR, which negatively regulates autophagy in well-fed organisms. Rheb and TSC1/2 control the activity of TOR, both acting downstream of PI3K and AKT. (B) Developmental autophagy as described in this chapter is triggered by ecdysone signaling that it is time to eliminate tissues or organs. Note that in this scenario activation of EcR by 20E inhibits PI3K, providing an opportunity for cross-talk between these signaling pathways. (For clarity, the steps in the pathway linking PI3K with autophagy are omitted in panel (B)). This form of autophagy can involve activation of known cell death genes, including caspases in some cell types. 20E, 20-hydroxyecdysone; AKT, a serine/threonine protein kinase; chico, *Drosophila* homolog of mammalian IRS1-IRS4, the insulin receptor substrate adaptor proteins; E93, an early ecdysone response gene that encodes a pipsqueak domain transcription factor; EcR, ecdysone receptor (nuclear hormone receptor); InR, *Drosophila* homolog of the mammalian insulin receptor; PI3K, phosphoinositide 3-kinase; Rheb, “Ras homolog enriched in brain,” a GTP-binding protein (GTPase); TOR, “target of rapamycin,” a serine/threonine kinase that belongs to the phosphoinositide-3-kinase-related kinase (PIKK) family of checkpoint kinases and is a part of a signaling pathway that regulates growth; TSC1/2, tuberous sclerosis complex proteins that function as GTPase activating proteins against Rheb; USP, *Drosophila* homolog of RXR that forms a heterodimer with EcR to form the functional insect ecdysteroid receptor. Adapted from Chang and Neufeld (2010).

Atg-family proteins that regulate the formation of the autophagosome (Ryoo and Baehrecke, 2010). This same pathway functions in *Drosophila*, with insulin-like peptides and insulin-like receptor corresponding to the mammalian insulin and insulin receptor, respectively. Other autophagy pathways have also been described in *Drosophila*. The first is a pathway in which autophagy results from an ecdysteroid signal (Figure 6); the second is part of the cellular response to oxidative stress in which Atg-family genes are transcriptionally activated by Jun-N-terminal kinase (JNK) signaling (Chang and Neufeld, 2010).

12.5.4.5. Autophagy and metamorphosis of neuromuscular systems A surprising phenotype is associated with mutations in *Drosophila* Atg-family genes (Shen and Ganetzky, 2010). In *Drosophila*, the number of boutons associated with each neuromuscular junction can be counted by immunolabeling synapses with markers such as anti-Synaptotagmin or anti-Nervous wreck. When bouton numbers were compared between wild type flies and flies with loss-of-function Atg mutations, the

mutants displayed significantly reduced bouton numbers, by up to 50%. Bouton numbers can be increased by pro-autophagic genetic manipulations. The site at which autophagic processes regulate normal development of the neuromuscular junction appears to be the motoneuron, rather than the muscle target (Shen and Ganetzky, 2009). Reduced expression of the Highwire protein appears to be instrumental in the regulation of synaptic development via autophagy. Highwire is an E3 ubiquitin ligase previously shown to reduce the formation of synapses (Wan *et al.*, 2000). The substrate specificity of autophagic pathways in motoneurons is astonishing. Whether this is a singular mechanism for the regulation of synaptic plasticity or the first of many “selective autophagy” targets remains to be discovered.

12.5.5. PCD of Muscles during Metamorphosis

There is extensive loss of the embryonically-derived musculature within the abdomen in *Drosophila* following adult eclosion (Miller, 1950; Kimura and Truman, 1990). Although some of the persisting muscles continue to

function during pupal life and adult emergence, others provide a template for adult myogenesis (Broadie and Bate, 1991; Bate, 1993). Some of these new muscles, including male-specific muscles, depend upon innervation for differentiation and survival (Bate, 1993). Phagocytosis of degenerated (also referred to as histolyzed) muscle is a feature of the larval–pupal transition in *Drosophila* (Crossley, 1978; Bate, 1993), but not of muscles that die following adult eclosion (Kimura and Truman, 1990; Jones and Schwartz, 2001).

In contrast with *Manduca*, relatively little is known about the genes involved in the death of *Drosophila* muscles during metamorphosis. A recent study has demonstrated that the nuclear spindle matrix scaffold proteins East and Chromator play antagonistic roles in the death of the dorsal oblique external muscles during metamorphosis in flies (Wasser *et al.*, 2007). The Chromator protein appears to act late in histolysis, and is required for the complete destruction of dying muscles. EAST, which physically interacts with Chromator, appears to delay the loss of structural integrity in the muscles. The post-eclosion death of abdominal muscles in *Drosophila* is dependent on the microRNA Let7C (Sokol *et al.*, 2008). Deletion of Let7C ablates muscle death, which can be rescued with ectopic expression. The mRNA targets for Let7C that are essential for muscle death have not yet been determined.

12.6. Insights from Other Tissues

Study of PCD during embryogenesis and metamorphosis outside of the neuromuscular system is a growth area in cell death research. Many tissues have been studied, including the fat body and the germ cell lineages. Of particular relevance are two tissues that undergo PCD during the early hours of metamorphosis: the larval salivary glands, and the larval midgut. This is because they have been used effectively to explore the coupling of ecdysteroid signals and PCD. They also illustrate the co-occurrence of apoptosis and autophagy, and the cell-specificity of PCD pathways.

12.6.1. Larval Salivary Glands

The salivary glands of *Drosophila* form during embryogenesis, and constitute the largest secretory organ in the fly body. During larval life the salivary glands secrete digestive enzymes, while at the end of larval life they secrete the “glue proteins” that attach the pupa to the substrate – typically, the wall of the culture vial. In response to the pre-pupal pulse of ecdysone secretion at 10–12 h post-pupariation, the larval salivary glands undergo PCD (Jiang *et al.*, 1997). This hormonal cue serves to upregulate the expression of a number of multiple pro-apoptotic proteins, including Reaper, HID, Dronc, and Dark (Jiang *et al.*, 1997). Some

of this transcriptional regulation is direct: for example, EcR/USP response elements have been identified in both the *rpr* and *dronc* promoters (Jiang *et al.*, 2000; Cakorous *et al.*, 2004). Much of this regulation, however, reflects the activation of a cascade of early response genes by 20E, including E74, Broad Complex (BRC), and E93 (Lee and Baehrecke, 2001; Yin and Thummel, 2005). The importance of this indirect regulation is shown by the fact that E93 loss-of-function mutants have severe deficits in salivary gland PCD (Lee *et al.*, 2000). Notably, ectopic Reaper expression does not kill the larval salivary glands before the middle of the third and final larval instar, as prior to this PCD is blocked by high levels of DIAP1 (Yin *et al.*, 2007). Resistance to Reaper is removed during the second half of the third instar by a CREB-binding protein (CBP) mediated downregulation of DIAP1 in response to a mid-third instar ecdysteroid pulse (Yin *et al.*, 2007). The role of CBP in steroid-regulated nerve and muscle PCD has not yet been assessed.

Detailed analyses of the morphology of the dying larval salivary gland cells and the accompanying gene cascades provided a compelling example of the co-existence of caspase activation and the formation of autophagosomes. Expression of p35 in salivary glands keeps the cells alive, but does not block the formation of autophagosomes (Lee and Baehrecke, 2001). Confirmation of the simultaneous co-activation of multiple PCD-related programs comes from microarray and SAGE-based studies of gene expression in dying larval salivary glands (Gorski *et al.*, 2003; Lee *et al.*, 2003). Among the several hundred transcripts identified in these studies were known apoptosis-related transcripts, genes associated with autophagy, and non-caspase proteases (Yin and Thummel, 2005).

PCD of larval salivary glands differs from the PCD observed in the embryonic and metamorphosing nervous system, in that the process in the salivary gland destroys an entire organ, whereas in the CNS selected individual cells die while their neighbors survive. PCD in salivary glands is more similar to the phenomenon observed in the ISMs. Whether the distinct selective nature of PCD activation in one case but not the others represents fundamentally or subtly different forms of regulation is unknown.

12.6.2. Larval Midgut

By the time the larval salivary glands begin to degenerate in response to the pre-pupal ecdysone pulse, the death of the larval midgut in response to the late third larval instar ecdysone pulse is already well underway (Lee *et al.*, 2002). However, in contrast to the salivary gland, midgut death occurs in a caspase-independent manner (Denton *et al.*, 2009). Instead, it is completely dependent on the autophagic pathway (Denton *et al.*, 2010). This model may represent the first clear demonstration of an autophagic cell death in the absence of a caspase-based component. Functional analyses of previously identified larval

salivary gland death genes were in turn identified as acting as 20E-dependent pro-survival or pro-death factors for the midgut (Chittaranjan *et al.*, 2009).

12.7. Summary and Conclusions

12.7.1. A Mature Field

The extent of research described in this chapter, much of it based on detailed genetic analyses of PCD in insect systems, indicates how thoroughly insect systems are embedded into the broader field of cell death studies. In addition to gaining a better understanding of how PCD matches insect neuromuscular systems to the stage of development, investigators are now linking the molecular machinery of PCD to their mechanistic analyses of the regulation of organ size, aging, and human neurodegenerative disease. Important pathways to watch in the future include the Hippo kinase pathway, which regulates the balance of cell proliferation and apoptosis during organogenesis (Badouel *et al.*, 2009); TOR-dependent autophagy and its relationship to lifespan (Bjedov *et al.*, 2010); and the regulation of cell death related genes by the forkhead box transcription factor FoxO1, which is in turn regulated by phosphorylation by the Parkinson's Disease-associated leucine-rich repeat kinase 2 (LRRK2) (Gandhi *et al.*, 2009; Kanao *et al.*, 2010).

12.7.2. Broad Phylogenetic Comparisons

Rapid strides in genome sequencing will ultimately result in numerous interesting comparative studies of the molecular machinery of all forms in cell death. There is already a wide range of insect genomes that have been sequenced, including 12 different *Drosophila* species and several other dipterans, as well as members of the Lepidoptera, Hymenoptera, Coleoptera, Hemiptera, and Phthiraptera. In addition, the genome of *Daphnia pulex*, a member of the crustacean sister group, has also been sequenced. These data provide a rich resource for comparative analysis of PCD pathways and PCD evolution. Studies of PCD in insects have already raised several pertinent questions, including the basis for distinct molecular palettes and pathways for regulating common cellular outcomes in divergent organisms.

12.7.2.1. Evolutionary scenarios for the *grim-reaper* gene complex The small Reaper, Grim, and Sickie proteins are clearly related, and the much larger Hid protein is also likely to share a common origin. Why are four related proteins needed to control DIAP1 inhibition and apoptosis, and how similar are the functions of these proteins? Gene complexes can provide enhanced capabilities for a cell to control specific biological processes by providing gene products that exhibit overlapping yet distinct expression patterns and functions. Presumably,

the existence of the *reaper*, *hid*, *grim*, and *sickle* genes in *Drosophila* enables cells to control apoptosis and other processes with the exquisite specificity and efficiency that are required for development. It will be fascinating to discover the functional differences that distinguish these proteins. Already, gene expression studies, as well as loss-of-function and gain-of-function mutants, have indicated that these genes exhibit distinct patterns of expression and non-redundant cell killing activities (see, for example, Grether *et al.*, 1995; Robinow *et al.*, 1997; Zhou *et al.*, 1997; Wing *et al.*, 1998, 2002).

While the *grim-reaper* genes are essential cell death activators in *Drosophila*, *bona fide* structural homologs are not found in *C. elegans* or vertebrates. Indeed, until recently, RHG genes had only been identified in other dipterans, including several *Drosophila* species, the blowfly *Lucilia cuprina*, and the mosquitoes *Anopheles gambiae* and *Aedes aegypti* (White *et al.*, 1994; Chen *et al.*, 2004; Zhou *et al.*, 2005). However, a recent bioinformatic analysis identified an RHG gene from the lepidopteran *Bombyx mori* (Bryant *et al.*, 2009). The predicted proteins share a highly conserved NH2-terminal RHG domain, and most also possess a GH3 domain/Trp block. These RHG proteins are likely to serve as PCD-promoting IAP antagonists. Interestingly, the mosquitoes and silkworm genomes each appear to possess a single RHG family member, as opposed to the four related, linked genes in *Drosophila*. This suggests that gene duplication events leading to the four linked RHG genes in *Drosophila* occurred recently, and that, in comparison with the caspases and Bcl-2 family proteins, there has been an extremely rapid divergence of the coding sequences of IAP-antagonists. It will be of interest to clarify the phylogenetic distribution of RHG genes.

12.7.3. Future Directions

It can be confidently predicted that studies of the diverse mechanisms of PCD in insect neuromuscular systems across the lifespan will lead to greater understanding of the molecular mechanisms of metamorphosis, particularly the ways in which developmental hormone signals are coupled to neuronal and muscle survival. Although studies of developmental PCD in insects now encompass other tissues, studies of PCD in the CNS will remain important because they offer the opportunity to study the life (and death) history of individual identified neurons. Studies of PCD in ISMs will continue to offer the challenge of segment-specific death phenotypes. Studies driven by investigators eager to identify the molecular pathologies involved in human neurodegenerative disease and human myopathies are likely to become an ever-increasing force in the field of insect PCD, particularly as researchers become ever more adept at transitioning seamlessly across mammalian and insect model systems (see, for example, Godin *et al.*, 2010). The focus on translational research,

however, should not obscure the many fascinating basic questions that remain unanswered concerning PCD and insect neurons and muscle. A partial list includes the following:

- Why are there so many PCD pathways? Can we develop a model that allows us to predict the PCD program a cell will select under specific developmental and environmental conditions?
- What are the downstream effects of PCD in neuromuscular systems? That is, do dying cells in turn produce signals that influence the phenotype and/or survival of neighboring cells? For example, it has been shown in fly wing that dying cells produce a caspase-dependent signal (wingless) that induces neighboring cells to proliferate. This phenomenon, referred to as compensatory cell proliferation, is thought to help maintain homeostasis in developing tissues (see, for example, Ryoo *et al.*, 2004). The relevance of this phenomenon to other organs and tissues is unknown.
- Is our view of insect PCD skewed because we rely so heavily on a single insect model? For example, there is growing interest in epigenetic regulation of apoptotic genes (Mazin, 2009; Hajji and Joseph, 2010; Jazirehi, 2010), yet the *Drosophila melanogaster* genome does not encode any methyltransferases – thus one key component of epigenetic DNA modification cannot be studied in this species, although other insect species such as the honey bee have methyltransferase enzymes comparable to those of mammals (Gabor Miklos and Maleszka, 2010).
- Do vertebrate-like neurotrophins function to regulate PCD in insect embryogenesis? During metamorphosis? The recent report describing *Drosophila* Neurotrophin 1 (DNT-1) should spark new interest in this possibility (Zhu *et al.*, 2008).
- What is the relationship of cell energy budgets to PCD in insect neurons and muscles?
- What role, if any, do microRNAs play in the regulation of nerve and muscle survival?
- Can genetic tools (RNAi, transfection, etc.) be applied to the study PCD in *Manduca* to facilitate the analysis of the novel cell death genes isolated from this species?

A persisting question in the field is the nature of the relationship between pathological cell death and PCD that is necessary for normal development.

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