Genetic control of programmed cell death in *Drosophila melanogaster*

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Abstract

Apoptosis is a genetically controlled form of cell death that is an important feature of animal development and homeostasis. The genes involved in the control and execution of apoptosis are conserved throughout evolution. However, the actual molecular mechanisms used by these genes vary from species to species. In this review, we focus on the genetic components of apoptosis in the fruit fly *Drosophila melanogaster*, and compare their mode of action to the one employed by the homologous genes in mammals. We also cover recent advances that show that apoptotic genes have a requirement in processes other than apoptosis.

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1. Developmental biology and apoptosis: an intimate association

The development of an organism into a fully functioning adult from a mass of undifferentiated cells is one of the most fascinating and complex processes in all of nature. Both commonplace and intricately regulated, the mechanisms controlling this process have been the subject of intense study stretching back to the early nineteenth century.

Fundamentally, developmental biologists all seek answers to the same basic question: How do living organisms coordinate complementary and sometimes antagonistic processes in order to produce a fully functioning adult form? The answers to these
and other questions have provided a wealth of information, much of which has directly impacted our understanding of the roots of diseases such as cancer [1]. And it is the study of programmed cell death, which provides perhaps the most intriguing question in all of biology: Why do cells die?

The notion that cells might die via a regulated, normal physiological process was first proposed by Kerr et al. [2-3] following analyses of ischemic liver tissue and adrenal and breast carcinoma samples treated with radiotherapy. In each case, they repeatedly observed a morphology that would later become associated with the canonical apoptotic morphology: disruption of cellular membranes, formation of small cytoplasmic masses and condensed nuclear chromatin, preservation of lysosomal integrity, followed by engulfment and degradation. This process was dubbed apoptosis [2]. Perhaps most importantly, Kerr and Wyllie believed this process was a normal physiological process and therefore one possibly controlled by a genetic program [4].

Regulation of apoptosis activation is an exquisitely controlled process, one which is critical to a wide variety of developmental processes, including removal of unnecessary anatomical structures, control of cell number, morphological sculpting, and elimination of injured or dangerous cells [5]. A readily visible, everyday example of an apoptotically-driven structural deletion is the elimination of the tadpole’s tail as it approaches adulthood. Similarly, the removal of inter-digital webbing from the toes of the human fetus and certain non-waterfowl birds is a vivid example of apoptosis in sculpting external morphology.

Cell number adjustment and elimination of injured/dangerous cells are critical to proper organism development and homeostasis. Cell number adjustment has a particularly important role to play within the developing nervous system. It has been estimated that during development, vertebrate embryos lose up to fifty percent of their neurons and other cell types programmed cell death [6]. This process is believed to occur in neurons, which fail to form synaptic connections with other neurons. In the developing Drosophila melanogaster central nervous system (CNS), programmed cell death figures prominently. Within the ventral midline, large numbers of neurons and glia cells are specified during mid-embryogenesis; however, at the end of embryogenesis a smaller cell number remains after successive rounds of apoptosis [6,7].

For non-developing cells, apoptosis is a critical component of tissue homeostasis, providing a mechanism for the elimination of dangerous and/or injured cells. Cells must receive a constant stream of survival signals generated by both the extracellular matrix and neighboring cells. Cultured cells, which have been removed from any source of exogenous survival signal will undergo apoptosis [8]. Furthermore, misregulation of cell death has been implicated in a wide variety of diseases exhibiting inappropriate suppression or activation of programmed cell death. Examples include autoimmune disorders, viral infections, neurodegenerative disease, leukemia, and cancer [9]. A classic cancer cell hallmark is the ability of an isolated cell in culture to survive despite the absence of any growth or survival signal. In addition, cells may acquire “renegade” status (i.e., become cancerous) through a variety of pathways: acquisition of limitless replicative potential, insensitivity to anti-growth signals, and acquisition of the ability to secrete growth and survival signals to neighboring cells [1]. Therefore, in mature tissues, the apoptotic elimination of potential renegades serves to protect the organism against potentially lethal consequences of their survival.

2. Drosophila programmed cell death: an overview

D. melanogaster provides an excellent model in which to study programmed cell death because cells in Drosophila exhibit the same developmental plasticity observed in humans. Practically, this means that the fruit fly allows an analysis of programmed cell death because cells in Drosophila study programmed cell death because cells in Drosophila's nervous system. It has been estimated that during development, vertebrate embryos lose up to fifty percent of their neurons and other cell types programmed cell death [6]. This process is believed to occur in neurons, which fail to form synaptic connections with other neurons. In the developing Drosophila melanogaster central nervous system (CNS), programmed cell death figures prominently. Within the ventral midline, large numbers of neurons and glia cells are specified during mid-embryogenesis; however, at the end of embryogenesis a smaller cell number remains after successive rounds of apoptosis [6,7].

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Fig. 1. Deletion of the H99 locus completely blocks apoptosis in Drosophila embryos. Acridine Orange staining reveals apoptotic cells. (A) Wild-type embryos containing the intact H99 locus (which include hid, grim, and rpr) experience widespread apoptosis, with the largest number of dying cells concentrated in the head region (arrowhead) and central nervous system along the ventral midline (arrows). (B) Deletion of the H99 locus completely blocks all embryonic programmed cell death. Note that sickle is intact but does not appear to affect the elimination of programmed cell death in these embryos. Acridine Orange figures taken from reference [11].

jafrac2, maps to a different location in the genome, but has similar characteristics to the H99 genes [17].

The domain structures of Rpr, Grim and Hid as well as a functional homolog in humans, Smac, are outlined in Fig. 2. They encode novel proteins without significant homology to other proteins in the database, and among each other they vary considerably in size. However, they share a common motif at the N-terminus, termed the RHG motif. This motif is critical for the pro-apoptotic function of the H99 proteins, and the functional significance of this motif will be discussed below (see Section 2.3).

Individual point mutations are only available for hid. Consistent with an important role in cell death, hid mutant embryos have reduced levels of PCD and contain extra cells in the head and CNS [6,12]. The genetic requirement of rpr was more difficult to assess. Attempts to isolate rpr mutants using chemical mutagens were unsuccessful [18]. However, it was possible to analyze the rpr mutant phenotype.

Fig. 2. Conservation of the RHG motif among H99 genes and the mammalian counterparts. Each of the pro-apoptotic genes located within the H99 locus (plus Ski and Jafrac2, not shown) and the mammalian counterpart Smac share a conserved N-terminal region, the RHG motif [78]. Outside of this motif they are not similar to each other and vary significantly in size. The RHG motif allows the Drosophila proteins to competitively bind the BIR2 domain of the inhibitory protein DIAP1, which releases DIAP1’s inhibition of the caspase DRONC. For more detail see text. Note that in each example, the RHG motif is located at the extreme N-terminal portion of the protein. In the case of Smac, the RHG is also located at the extreme N-terminus after the mitochondrial localization sequence is cleaved off in the process of mitochondrial translocation. Not drawn to scale.
by combining two chromosomal deficiencies (XR38/H99), which in trans delete the rpr locus, but leave the hid, grim and sickle genes intact. Surprisingly, XR38/H99 animals are viable and embryonic PCD proceeds normally [18]. However, XR38 and sickle suggest that in the absence of function of the adult brain in these mutants. Subsequent analysis of these neuroblasts shows that the H99 genes are not physiologically equivalent, although they may use similar mechanisms to induce apoptosis.

2.1. Caspases

What sets apoptosis apart from necrosis is the implementation of caspases, which are conserved across a wide range of species, from worms to mammals [19]. These cysteine proteases target and cleave their substrates specifically after an aspartate residue, and act as the executioners of the cell suicide program. Caspases are widely expressed as inactive proenzymes precursors and consist as such of a prodomain of variable length (see below), followed by a large (p20) and a small (p10) unit (Fig. 3) which contain the residues essential for substrate recognition and catalysis. Activation of caspases occurs either through recruitment into large protein complexes (see below) or by proteolysis, separating the p20 and p10 subunits, allowing their reassembly as an active heterotetramer. Once unleashed, they ruthlessly break down the variety of cellular complexes, including cytosolic proteins, cytoskeletal elements, and nuclear lamins [19].

To date seven caspase-encoding genes have been identified in the Drosophila genome: dcp-1, dredd, drice, dronc, decay, strica, and damm ([23–29]; reviewed in [30,31]). Based on the prodomain structure, Dcp-1, Drice, Decay, and Damm belong to the effector caspase category. Dronc (Drosophila Nedd-2 like Caspase) and Dredd (death related CED-3/Nedd2-like protein) can be classified as apical caspases. Dronc contains a CARD motif in the prodomain (Fig. 3), whereas Caspase-8 is characterized by the presence of a long N-terminal prodomain (Fig. 3), and is most similar to mammalian Caspase-9 into the DISC (death inducing signaling complex) via DED–DED interaction with its adaptor FADD (not illustrated) [20–22]. Effector caspases either have a shortened or absent N-terminal prodomain (Fig. 3), and carry out the destruction of the cell by targeting a wide variety of cellular complexes, including cytosolic proteins, cytoskeletal elements, and nuclear lamins [19].

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Caspases are divided into two general classes: apical and effector. During the apoptotic process, caspases are activated in an amplifying proteolytic cascade, cleaving one another in sequence. As their name implies, apical (or Class I) caspases lay near the top of the proteolytic hierarchy and are activated at the onset of apoptosis. Upon activation, the apical caspases activate the downstream effector (or Class II) caspases via proteolytic processing [19]. Apical caspases are characterized by the presence of a long N-terminal prodomain. The long prodomain is important to the regulation of caspase activity as it contains several homotypic protein–protein interaction motifs. For example, Caspase-9 contains the CARD motif (caspase activation and recruitment domain; see also Fig. 3), whereas Caspase-8 is characterized by the presence of the DED motifs (death effector domain). These motifs are crucial for recruitment of the apical caspase into appropriate death complexes: Caspase-9 into the apoptosome via CARD–CARD interaction with its adaptor protein Apaf-1 (Fig. 4) [20–22]; Caspase-8 into the DISC (death inducing signaling complex) via DED–DED interaction with its adaptor FADD (not illustrated) [20–22]. Effector caspases either have a shortened or absent N-terminal prodomain (Fig. 3), and carry out the destruction of the cell by targeting a wide variety of cellular complexes, including cytosolic proteins, cytoskeletal elements, and nuclear lamins [19].

Dronc, Drice, and Decay are characterized by the presence of a long N-terminal prodomain (Fig. 3), whereas Caspase-8 is characterized by the presence of a long N-terminal prodomain (Fig. 3), and is most similar to mammalian Caspase-9 and -1, while Dredd is structurally most similar to Caspase-9 [25,26]. Although Dredd’s prodomain contains a pair of DEDs, most available evidence points to a non-apoptotic role in the regulation of the Drosophila immune response rather than programmed cell death [32]. In addition, its expression is not widespread like most other caspases, nor is ectopic expression sufficient to induce apoptosis [25].

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![Fig. 3. Domain structures of selected pro- and anti-apoptotic proteins in Drosophila. Shown are the domain structures of Dronc, Drice, Dap1, and dBruce.](image-url)

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Fig. 4. Comparison of the cell death pathways in Drosophila and mammals. In Drosophila, the IAP antagonists Rpr, Hid and Grim release Dronc from Diap1 inhibition through induced self-ubiquitination (ub) of Diap1 via the RING domain. Free Dronc can then associate with the adaptor protein Dark to become activated. Active Dronc proteolytically processes the effector caspases Drice and Dcp-1 and induces apoptosis. In mammals, release of Cytochrome c (CytoC) from mitochondria activates the adaptor protein Apaf-1 with recruits and activates proCaspase-9 into the apoptosome. XIAP inhibits processed Caspase-9 through binding to the small subunit. Smac, also released from mitochondria, releases active Caspase-9 from XIAP inhibition through ubiquitination. For more details see text.

Although Strica bears a long prodomain, it does not contain any of the known protein-protein interaction motifs, such as CARD or DED [28]. However, a phylogenetic analysis shows that it clusters with effector caspases [33]. Mutants in strica, which could help to clarify the genetic role of this caspase are currently not available. Thus, with the role of Strica still undefined, it appears that Drosophila contains only one classical apical caspase, Dronc.

Dronc’s role in PCD regulation has been well established. Consistent with its function as an apical caspase, Dronc can cleave and activate the effector caspase Drice in vitro [34,36]. Dronc expression is increased in response to developmental pro-apoptotic cues such as increased titers of the steroid hormone ecdysone during metamorphosis [26], and recent evidence indicates that this may occur via direct binding of the ecdysone receptor to the Dronc promoter [35]. Overexpression of dronc in the developing fly eye induces cell death and tissue loss [36,37]. Dominant negative constructs and RNAi experiments support a role for dronc in developmental cell death [36,37]. Ablation of Dronc expression using RNA interference techniques largely suppressed Diap1 loss of function-induced apoptosis, and also inhibited processing of the effector caspase Drice following stimulation of apoptosis in Schneider cells, a Drosophila cell line [38]. Recently, inactivating mutations of the dronc gene have been reported allowing for a genetic analysis of dronc [39–41]. These studies establish Dronc as an essential caspase for PCD in Drosophila. However, it was also demonstrated that not all developmental PCD is dependent on Dronc. The nature of the dronc-independent cell death pathway is currently unknown.

2.2. Inhibitor of apoptosis proteins (IAPs)

In mammals as well as Drosophila, caspases are normally biochemically inactivated by binding to inhibitor of apoptosis proteins [42]. IAPs are a highly conserved class of proteins with members in all eukaryotic species including humans, Drosophila, C. elegans and yeast [43]. IAPs were originally discovered in Baculovirus due to their ability to functionally substitute for the baculoviral p35 protein, another apoptosis inhibitor [44–46]. It is believed that baculoviruses express IAPs to escape the apoptotic host response, which would otherwise limit viral replication.

IAPs share several structural motifs. The presence of between one and three tandem baculoviral IAP repeat (BIR) motifs qualifies for membership in the IAP family [42]. The BIR motif, a ~80 residues comprising Zn-binding fold, is essential for the anti-apoptotic function of IAPs (see below). In addition to the BIR motif, some IAPs contain a RING (Really Interesting New Gene) domain [47] implying that ubiquitin-mediated protein degradation is involved in IAP function. As discussed below, targets of RING-mediated ubiquitination are caspases, the H99 proteins and IAPs themselves. The most unusual IAP is the Apollon/Bruc protein: it encodes a gigantic protein of more than 4830 residues with a single BIR domain at the N-terminus and a ubiquitin-conjugating (ubc) motif at the C-terminus [48], providing further evidence that ubiquitin-mediated degradation is involved in IAP function (for detailed review about the proteolytic activity of IAPs see reference [49]).
IAPs appear to interact with only a certain subset of caspases. For instance, mammalian XIAP, c-IAP-1, and c-IAP-2 can bind to and inhibit Caspase-3, -7 and -9 but fail to interact with Caspase-1, -6, -8 and -10 [50,51]. Survivin binds specifically to Caspase-3 and -7, but not to Caspase-8 [52]. NAIP has been shown to bind to Caspases-3 and -7 [53]. The interaction of IAPs with caspases requires the BIR motifs of IAPs. The BIR motifs of those IAPs with more than one BIR motif fold into functionally independent structures. Interestingly, the individual BIR motifs of a given IAP have different substrate specificity. For example, XIAP contains three BIR domains. However, inhibition of Caspase-3 and -7 by XIAP requires the BIR2 domain and a small segment amino-terminal to BIR2 [54–58], whereas BIR3 inhibits Caspase-9 [59,60]. Similarly, BIR1 of Drosophila IAP1 (Diap1) was found to specifically inhibit Dcp-1 and Drice, whereas BIR2 inhibits Dronc [61,62,67]. Thus, the presence of several BIR motifs increases the flexibility with which an IAP inhibits caspases. In addition, IAP binding to caspases requires – paradoxically – cleavage of caspases [54–60].

The only known exception is Drice, which is inhibited through binding of Diap1 to the prodomain of the zymogen form of Dronc (see also Section 3) [36,61,62].

The Drosophila genome encodes four IAPs of which the diap1 gene appears to be most important and is the subject of intensive analysis. diap1 encodes for an IAP with two BIR and one RING domains (Fig. 3) [63]. Loss of diap1 leads to uncontrolled caspase activation causing massive and widespread apoptosis with an early onset resulting in embryonic lethality [64–66] suggesting that diap1* has an essential genetic requirement for the inhibition of apoptosis. Consistent with this notion, Diap1 has been shown to directly interact with at least three Drosophila caspases, the apical caspase Dronc and the effector caspases Drice and Dcp-1 [36,61,62,64,67].

In addition to loss-of-function mutations, diap1 can also mutate to obtain gain-of-function character. These gain-of-function mutations protect cells better from apoptosis than does the wild-type gene [65,66,68], and can be classified as ‘Super-IAPs.’ Interestingly, they behave as ‘Super-IAPs’ in a dominant manner, i.e. the gain-of-function mutation of only one of the two genomic copies is sufficient to confer the ‘Super-IAP’ activity [65,66,68]. Molecular analysis of these ‘Super-IAPs’ reveals that they affect either one of the BIR [65] or the RING domains [66,68]. Subsequent biochemical analysis demonstrated that the diap1 mutations affecting the BIR domains diminish the interaction with the H99 proteins [65] (see below), whereas the RING domain mutants block the E3 ligase activity of Diap1 [66,68]. As a consequence, auto-ubiquitination and degradation of the mutant Diap1 is blocked and the cell death signal is not propagated in these mutants.

Not much information is available for the remaining three IAPs in Drosophila, Diap2, Deterin and DBruce. However, mutations in DBruce, the Drosophila homolog of mammalian Apollon/Brice (Fig. 3), have been isolated as enhancers of rpr-induced apoptosis [69], and based on the presence of the ubc domain it is possible that dBruce assists Diap1 with the ubiquitination of substrates. Interestingly, dBruce mutations do not influence hid-induced apoptosis [69], suggesting that rpr and hid might utilize different mechanisms to promote apoptosis.

2.3. The ‘Gas and Brake’ model

Programmed cell death in Drosophila is an intricate affair involving the aforementioned H99 genes, caspases, and IAPs (Fig. 4). In cells which have not received a death signal, the BIR2 domain of Diap1 binds to the prodomain of Dronc [61,62], and this prevents the association of Dronc with the Apaf-1 homologue Dark, also known as Hac-1 or dApaf-1 [70–72]. The Diap1–Dronc interaction enables the RING domain of Diap1 to tag Dronc for degradation via ubiquitin-mediated proteolysis [68], and thus provides an efficient means to inactivate Dronc.

Dronc activation requires inhibition of complex formation with Diap1 [34,36,62]. The pro-apoptotic H99 proteins, Rpr, Hid, Grim, Skl, and Jafra2, bind with high affinity to the same BIR2 domain of Diap1 that is required for interaction with Dronc (Fig. 4) [61]. Thus, the H99 proteins compete with Dronc for binding to the BIR2 domain of Diap1, and are often referred to as IAP antagonists. However, it is not clear whether this competition results in displacement of Dronc from Diap1 inhibition or whether the H99 proteins bind and inactivate free Diap1, allowing newly synthesized Dronc to associate with its adaptor Dark to become activated. In either case, binding of the H99 proteins to Diap1 changes the substrate specificity of the RING domain, such that Diap1 self-ubiquitinates and is targeted for proteasome-mediated degradation [73–75]. The H99 proteins might be degraded in the process [76,77]. Thus, activation of PCD in Drosophila involves the relief of the IAP-regulated caspase repression by the H99 pro-apoptotic proteins (Fig. 4).

The secret to the pro-apoptotic ability of the H99 proteins lies in the fact that, while otherwise completely dissimilar, all five share a highly conserved N-terminal motif known as the RHG motif (rpr, hid, grim; Fig. 2) [78], also known as IBM (IAP binding motif). It is this motif, also found in the mammalian ortholog Smac [79] that binds to the BIR2 of Diap1, and competitively inhibits Diap1 from binding to the apical caspase Dronc [61]. Thus, released from Diap1’s repressive embrace, Dronc is free to form a complex with its adaptor protein Dark via CARD–CARD interactions and begins the killing spree for which it is so exquisitely designed.

The realization that induction of developmental apoptosis in Drosophila required the release of caspase inhibition led to the development of the “Gas and Brake” model for cell death (reviewed in reference [31]). Caspases are the “loaded gun” pointed at the cell, ready to wreak havoc, death, and destruction upon the cell. The cell is not completely helpless and employs a “brake” upon caspase activation in the form of IAP proteins. However, binding of any of...
3. Comparison of the cell death pathways in Drosophila and mammals

Despite the similarities of the cell death pathways employed in Drosophila and in mammals, there are also a few notable differences. First, mammalian caspases are inhibited by IAPs after the active form of the caspase has been generated. For example, after proteolytic separation of the p20 and p10 subunits of Caspase-9, the p10 subunit exposes at its newly generated N-terminus a motif similar to the RHG motif in the Drosophila H99 proteins [59]. This motif is used by XIAP to bind to and inhibit Caspase-9 in its processed form. Whereas Drice and Dcp-1 are also inhibited in their processed forms [61], this does not apply to the apical caspase Dronc. Here, inhibition occurs through binding of Diap1 to the prodomain of the zymogen form of Dronc [36,61,62]. This difference implies that Diap1-mediated inhibition of Dronc processing plays a much more important role for PCD regulation in Drosophila compared to mammals.

Second, in mammals, an essential component for the transduction of the cell death signal is Cytochrome c [80,81]. Cytochrome c is needed for activation of the adaptor protein Apaf-1, which is required for apoptosome formation (Fig. 4). Usually present in mitochondria, Cytochrome c is released into the cytosol in response to apoptotic signals. The release of Cytochrome c is regulated by pro- and anti-apoptotic members of the Bcl-2 protein family. In Drosophila, no conclusive evidence has been found to date that demonstrates a similar role for Cytochrome c [82,83].

S2 cells depleted of Cytochrome c by RNA interference (RNAi) still respond to apoptotic cues [84,85]. Rather, it appears that the Apaf-1 homologue Dark does not require Cytochrome c for activation. It has been proposed that free Dronc is constitutively activated through binding to Dark [38]. Two Bcl-2 family members have been identified in Drosophila [86–89], however, their precise role in regulation of developmental PCD and Cytochrome c release is unknown due to lack of genetic mutations.

Finally, the fundamental role of Diap1 for caspase regulation and PCD in Drosophila stems from the analysis of its mutant phenotype. Homozygous diap1 mutant embryos display a dramatic cell death phenotype: essentially every cell is apoptotic [64–66]. These embryos die during early embryogenesis. In contrast, gene inactivation studies of XIAP in the mouse fail to reveal any striking phenotype [90], although the loss of XIAP might be compensated for by other IAPs such as c-IAP1 and c-IAP2. This is consistent with the observation that expression levels of c-IAP1 and c-IAP2 are up-regulated in XIAP−/− animals [90]. A similar difference was detected at the level of the H99 genes. Homozygous H99 mutant embryos completely lack developmental PCD [11], whereas homozygous knockout animals of diablo, the murine homologue of Smac, are normal without detectable phenotype [91].

4. Caspases: surprising non-apoptotic functions

Recent evidence has demonstrated that caspase function is not solely limited to cell death execution. As already mentioned earlier, Dredd is involved in the immune response in Drosophila [32]. Newly discovered roles include facilitation of sperm differentiation, border cell migration, and even cell proliferation.

4.1. Function in sperm differentiation

Sperm differentiation in Drosophila requires that the developing spermatozoa undergo a process known as individualization, whereby most of the cytoplasm is expelled [92,93]. Arama et al. found that this process required active Drice [94]. In addition, they noted that expression of the active form of the effector caspase Drice was detectable at the onset of individualization, and that p35 (an inhibitor of effector caspase activity) was sufficient to block this process. Taking these findings further, Huh et al. were able to demonstrate that Dronc activation occurred at sites of spermatid individualization [94]. Overexpression of Diap1, or of a dominant-negative mutant of Dronc, as well as Dark RNAI were used to inhibit caspase activation. Furthermore, they showed that all three proteins were required for the process to function normally [95]. Finally, Dredd and its adaptor dFadd were both required to complete the individualization process.

What is most remarkable from these findings is that mechanisms so well suited to killing cells seem to have been harnessed to further the individualization process. Huh et al. suggest that caspases may cleave specific target proteins, which promote the process [95]. However, it is not known at this time what these cleavage targets might be.

4.2. Function in border cell migration

Spermatogenesis is not the only sexual development process influenced by the PCD machinery. In an elegant genetic analysis, Giesbrecht and Montell were able to establish that...
Diap1 was necessary for proper migration of border cells, and that this activity involves the cooperation of the small GTPase Rac1 [96]. Drosophila oogenesis utilizes several cell types throughout the process. The egg chamber is composed of an oocyte and a collection of fifteen nurse cells, all of which are surrounded by a monolayer of follicular epithelial cells. Within each egg chamber are two specialized follicular cells, known as polar cells. One cell develops at the posterior end of the egg chamber while the other forms at the anterior region. The anterior polar cell recruits a variable number (from 6 to 8) of neighboring cells to form the border cell cluster. During oocyte development, this cluster migrates the border region, which lies between the developing oocyte and nurse cells.

Previously, the Montell group had shown that expression of a dominant-negative Rac1 (Rac1<sup>N17</sup>) in the border cells inhibited the cluster’s migration [97]. However, Giesbrecht and Montell found that overexpression of Diap1 along with increased G-Actin production was sufficient to rescue the migration defect, and that diap1 mutants affecting the BIR domains exhibited defects in border cell migration similar to Rac1<sup>N17</sup> while diap1 mutants affecting the RING domain did not [96]. The authors demonstrated the failure of p35 overexpression to suppress the border cell migration defect in the Rac1<sup>N17</sup> background. Pulldown assays performed in Drosophila S2 cells demonstrated that Rac1, Profilin, and Diap1 associate in a nucleotide-independent manner [96]. It remains to be seen whether Diap1’s effect upon Rac1-dependent migration is a function of its role in repressing Dronc activation, or whether this effect relies on some other mechanism.

### 4.3. Function in cell proliferation

Cell proliferation and cell death during development is often thought of as a zero-sum enterprise, with unlucky cells being pruned away to fine-tune the developmental process. Yet researchers have long been aware that in the fly wing disc, irradiation-induced cell loss is followed by compensatory cell proliferation, the result of which is a nearly normal adult wing [98]. Two recent studies came to the conclusion that the non-autonomous proliferation signals driving this post-apoptotic growth derive from the dying cells themselves. However, they somewhat disagree about the mechanistic details how this might happen [99,100].

Both studies showed that proliferation levels in cells in the posterior compartment of the wing expressing both Hid and the caspase inhibitor p35 were 2–4 times greater than those expressing p35 alone [99,100]. Importantly, most of the cell proliferation occurred in cells outside of the Hid and p35 expressing area, suggesting that a signal originates in the dying cells that stimulates the proliferation of cells neighboring dying cells. This compensatory proliferation might replace the dying cell(s) and maintain appropriate homeostasis of the tissue. However, the mechanism, which induces the proliferative signal in dying cells is subject of controversy. When Huh et al. co-expressed a dominant-negative form of Dronc in cells already expressing Hid and p35, they found that the proliferative activity was reduced significantly [99]. Moreover, Dronc activation alone was sufficient to trigger increased cell proliferation in this domain [99], suggesting that Dronc is required for this activity. Ryoo et al. performed similar experiments and obtained different results [100]. In this case, the expression of a dominant negative Dronc mutant did not affect the capacity of dying cells to send out the proliferative signal. On the other hand, Ryoo et al. provided evidence that diap1 is important for this process [100], whereas Huh et al. concluded that diap1 is not involved in compensatory proliferation [99].

Another difference, which need to be addressed in the future, both studies reported a potential candidate for the secreted signal which is wingless (wg). Ryoo et al. also implicated the TGF-β homolog dpp as a candidate for the proliferative signal [100]. Both genes are known to have mitogenic potential in at least some developmental context [101,102]. Ryoo et al. also showed that activation of the JNK pathway in dying cells can induce the expression of wg and dpp, and non-autonomous cell proliferation [100]. Taken together, these results suggest that dying cells can induce compensatory proliferation, and that both wg and dpp may be the mediators of this process. The mechanistic details are unclear at this time, but will be addressed in the near future.

### 5. Conclusions

It has been over a decade after the discovery of rpr, the first RHG gene in <i>Drosophila</i> [11]. Since then we have seen a staggering increase in our knowledge of the molecular players of the apoptotic machinery in both flies and mammals. These studies revealed many similarities but also differences between flies and mammals. In the future it will be important to investigate how cell death is integrated in the complex organization of a developing organism. We also summarized results, which indicate that apoptotic components might be involved in a variety of processes other than apoptosis, and this list will likely grow in the coming years. Molecular genetic studies in <i>Drosophila</i> will continue to be at the forefront of these challenges, and will provide important information relevant to human health. After all, that is what genetic model organisms are good for.

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References


Hay BA, Wassarman DA, Rubin GM.


Boy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC. The c-IAP1 and c-IAP2 proteins are direct inhibitors of specific caspases. EMBO J 1997;16:6191–24.


