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Pathways regulating apoptosis during patterning and development

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Abstract

The patterning and development of multicellular organisms requires a precisely controlled balance between cell proliferation, differentiation and death. The regulation of apoptosis is an important aspect to achieve this balance, by eliminating unnecessary or mis-specified cells which, otherwise, may have harmful effects on the whole organism. Apoptosis is also important for the morphogenetic processes that occur during development and that lead to the sculpting of organs and other body structures. Here, we review recent progress in understanding how apoptosis is regulated during development, focusing on studies using *Drosophila* or *C. elegans* as model organisms.

Introduction

Apoptosis is the most widely studied form of programmed cell death and is characterized by a variety of morphological and biochemical aspects, such as the condensation of the nucleus and cytoplasm, the activation of proteases (caspases) and nucleases, that respectively degrade cellular proteins and DNA, and the fragmentation of apoptotic cells into membrane-bound bodies that are rapidly phagocytosed by neighboring cells. These aspects of apoptotic cell death make it distinct from necrosis, a form of cell death resulting from overwhelming cellular injury, in which cells swell and lyse, releasing their cytoplasmic contents into the extracellular space [1,2,3].

The nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* have been instrumental in defining the genetic and molecular pathways that regulate apoptosis during development. In brief, execution of apoptosis in these organisms requires the activation of caspases, a class of cysteine proteases that are constitutively expressed in virtually all cells as inactive zymogens. Upon death inducing signals, the inactive caspases are cleaved at specific aspartic acid residues, resulting in the removal of an inhibitory N-terminal domain and production of a large and a small subunit. These subunits then associate as a hetero-tetramer (2 large/2 small) to form the active protease that cleaves many cellular targets, leading to the apoptotic death of the cell.

The regulation of caspases occurs mainly by two distinct general mechanisms, employing regulatory cascades designed to either activate or inhibit caspases (Figure 1). In the “classic pathway”, derived from studies in *C. elegans* and mammalian systems, activation of the initiator caspase (caspase-9) results from the formation the apoptosome, a multi-protein complex containing CED-4/Apaf-1 and Cytochrome C (in mammals). This pathway is also regulated by the Bcl-2 family of proteins, which can facilitate or prevent the release of

Cytochrome C from mitochondria to the cytoplasm. A second pathway, largely derived from work in insects, is an inhibitory one, where caspase activation is blocked by Inhibitor of Apoptosis Proteins (IAPs). In this case, the activation of apoptosis requires the action of IAP antagonists, such as *reaper*, *hid* and *grim* in *Drosophila* [4–6]. It appears that both pathways are used in coordination to control the activation of caspases, but the relevant contribution of either branch varies according to the particular cell type and signaling paradigm [3,7–10].

Transcription is important

Many regulatory steps of the apoptotic process occur at the post-translational levels, either by protein-protein interactions and protein cleavage or by ubiquitin-dependent protein degradation [11]. Although all proteins required for the execution of apoptosis are constitutively expressed in virtually all cells, it has also been known for a long time that the induction of apoptosis often requires *de novo* transcription and protein synthesis [2,12,13].

Work in *C. elegans* has highlighted the importance of transcriptional regulation of the core apoptotic genes. The transcriptional upregulation of *egl-1* promotes cell death by antagonizing the interaction between CED-9 (Bcl-2 like) and CED-4 (Apaf-1 like) and allowing the release of CED-4 from mitochondria [14,15]. In the hermaphrodite specific neurons (HSN), the transcription factor TRA-1 directly binds the *egl-1* promoter to repress *egl-1* transcription and induce apoptosis of these cells in the males [16]. Recent work showed that the Hox gene *mab-5* is also required for the regulation of *egl-1*, and induction of apoptosis in the P11 and P12 cell lineages that generate neurons in the posterior ventral nerve cord. In the P11 lineage, MAB-5 forms a complex with the Pbx homologue CEH-20 to directly regulate *egl-1* transcription [17].

But *egl-1* is not the only cell death gene for which regulation at the transcriptional level is important. The correct timing of onset of death of the tail-spike cell requires transcriptional up-regulation of the *ced-3* caspase [18]. In this case, EGL-1 and CED-9 play only a minor role, and cell death occurs shortly after the onset of *ced-3* expression. The transcription factor PAL-1 (homologue of the mammalian tumor suppressor Cdx2) plays an important role by binding to the *ced-3* promoter to direct *ced-3* expression [18]. Also, *egl-38* and *pax-2*, members of the Pax2/5/8 class of transcription factors, act as modulators of *ced-9* transcription both in somatic and germline cell death [19].

One of the strongest evidence that new gene transcription is required for activation of apoptosis during normal development has come from *Drosophila*, where Reaper-family proteins are transcriptionally activated in doomed cells prior to their death [4]. More recently, it was also shown that expression of the apical caspase Dronc and the effector caspase Drice are upregulated in response to ecdysone [20,21], a hormone which induces apoptosis to eliminate the larval tissues during the process of metamorphosis. The transcriptional levels of *thread* (which encodes *Drosophila* Inhibitor of Apoptosis Protein 1 – Diap1) are also regulated by the Hippo signaling pathway, through the phosphorylation and inactivation of the transcriptional activator Yorkie [22] (see below). It appears that these recently discovered phenomena contribute to determine the general susceptibility of a cell towards apoptosis, but that additional regulation is needed for the acute initiation of cell death.

Fat signals to the Hippo pathway

Genetic screens in *Drosophila*, designed to identify mutants with excessive cell proliferation and hyperplastic growth, led to the identification of several genes acting in the Hippo signaling pathway [23,24]. These include two serine/threonine kinases, Hippo (Hpo) and Warts (Wts)/Lats, two adaptor proteins, Salvador (Sav) and “Mob as tumor suppressor” (Mats), and the transcriptional activator Yorkie (Yki). Wts phosphorylation and activation by Hpo is facilitated

by Sav and Mats. Activated Wts phosphorylates and inactivates Yorkie (Yki). Loss of Hpo, Wts, or Sav function or overexpression of Yki lead to up-regulation of *cyclin E* and *diap-1* and an increase in cell proliferation and inhibition of apoptosis.

Recent studies link the membrane protein Fat with the Hpo pathway [25,26,27,28]. Fat is a large protocadherin previously shown to function in the establishment of planar cell polarity, proximal-distal patterning of appendages, and to act as a tumour suppressor, restricting the growth of imaginal discs during larval development. Clones of *fat* mutant cells, as in clones of mutants of the Hpo pathway, overgrow due to increased proliferation and suppression of apoptosis and *cyclin E* and *diap1*, which are upregulated in Hpo pathway mutants, are also upregulated in *fat* mutants.

The precise molecular mechanisms linking Fat and the Hpo pathway are not known yet, but it seems Fat can regulate the Hpo pathway in more than one way. First, Fat can regulate Wts protein stability via the unconventional myosin Dachs, which directly binds Wts [25]. Second, Fat can interact with the Hpo pathway through the regulation of Expanded (Ex) [26–28]. Ex and the related protein Merlin (Mer) are members of the FERM (4.1, Ezrin, Radixin, Moesin) domain superfamily of proteins and act as upstream regulators of the Hpo pathway [29]. Fat co-localizes with Ex at the apical adherens junctions and is required for Ex, but not Mer or Moesin, apical localization [26–28]. It is not known how Fat regulates Ex apical localization or how Ex and Mer influence Hippo signaling, but it has been suggested that Fat might bring Ex together with Hpo and thereby activate this pathway [27]. Attempts to coimmunoprecipitate Fat and Exp failed, but Fat is a large (560 kDa) protein, which may be difficult to detect by coimmunoprecipitation assay [27]. On the other hand, Ex and Mer regulate endocytosis, and the steady state levels of several membrane receptors, such as Fat, Notch, EGFR, Patched and Smoothened are altered in Ex/Mer double mutants [30]. Hence, it is also possible that regulation of Hpo signaling by Ex/Mer occurs in an indirect manner, via the regulation of endocytosis of Fat. Additional experiments are required to address this possibility and the exact biochemical interactions between Fat, Ex and the Hpo pathway. Interestingly, cells mutant for *vps25*, a component of the ESCRT (Endosomal Sorting Complex Required for Transport), have higher levels of Hpo signaling [31], which points to the importance of endocytosis for the regulation of the Hpo pathway.

Another relevant finding was the identification of the microRNA *bantam* (*ban*) as a target of Yki and the Hpo pathway [32,33]. *ban* was previously identified as a developmentally regulated microRNA that induces cell proliferation and prevents apoptosis through the downregulation of the apoptosis-inducing gene *hid* [34]. In addition to *ban* other microRNAs, such as miR-14, miR-278 and the miR-2 family, play important roles as regulators of cell death genes [35,36].

Compensatory proliferation and cell competition

In developing tissues, cells that undergo apoptosis in response to stress or injury can induce proliferation of neighboring progenitor cells [37,38,39]. This regenerative phenomenon, termed “compensatory proliferation”, permits the organism to compensate for the loss of large numbers of cells and to develop normally patterned and sized organs in these situations. Significantly, doomed *Drosophila* imaginal disc cells produce proteins with known mitogenic and morphogenetic activities, such as Wnts and BMPs [38,39]. Furthermore, Wnt signaling is required for cell proliferation in this system [39]. Given the role of Wnt signaling for self-renewal of stem cells and in tumorigenesis in mammals, a similar mechanism may operate in vertebrates and there is considerable interest in elucidating this process in more detail.

New findings clearly establish the central role of the apical caspase Dronc in the induction of ‘compensatory proliferation’ [40]. Dronc mutants, but not Drice mutants, suppress compensatory proliferation induced by γ -irradiation or by expression of apoptotic proteins.

These findings establish a bifurcation downstream of Dronc in the regulation of apoptosis and “compensatory proliferation”. One branch leading to Drice cleavage and execution of apoptosis, and a second branch, independent of Drice, leading to the induction of “compensatory proliferation” [40]. Interestingly, p53 appears to be an important component of this second branch downstream of Dronc [41]. p53 is required for the induction of compensatory proliferation and is transcriptionally activated in “undead cells” by a mechanism that requires Dronc function [41]. Further investigations will certainly focus on the mechanism by which Dronc activates p53 transcription.

Cells can also undergo “compensatory apoptosis”, when rapidly proliferating cells induce apoptosis of their neighbors. For example, *Drosophila* cells expressing high levels of the proto-oncogene Myc proliferate faster and induce apoptosis in neighboring cells with lower levels of Myc [42,43]. This property is known as “cell competition” and is thought to occur due to the competition between fast-proliferating (Myc positive) and neighboring cells for growth and survival signals. Decapentaplegic (Dpp) seems to be an important survival signal in this context, since experimental activation of Dpp signaling in the out-competed cells (low Myc) can rescue these cells from apoptosis [43]. In addition, Dpp has also been associated with “morphogenetic apoptosis” [44], which occurs when there is an experimentally induced disruption of a Dpp signaling gradient between juxtaposed cells. Sharp boundaries of Dpp signaling during normal development can induce reaper-dependent apoptosis to sculpt the joint in the *Drosophila* leg [45] (see also “Patterning signals and proliferation in *Drosophila* imaginal discs” by Nick Baker in this issue).

Caspases without death

While it has long been known that certain caspases have non-apoptotic functions, a number of recent studies have illustrated the role of “apoptotic” caspases in cell differentiation or cellular morphogenesis [46,47]. For example, caspase activity is required for the removal of the bulk cytoplasm from developing spermatids in *Drosophila* [48]. Although caspase activation in this system does not lead to the death of the entire cell, sperm individualization resembles apoptosis in the sense that many cellular structures are degraded. Notably, the activation of apoptotic effector caspases in this system strictly requires a testis-specific form of Cytochrome C [48, 9]. This contrasts with the finding that Cytochrome C appears to only accelerate developmental apoptosis in the *Drosophila* retina [10].

Apoptotic proteins can also help alter the shape of neurons by dendrite pruning, the local degeneration of dendrites that occurs during *Drosophila* metamorphosis. Dendrite pruning requires the degradation of Diap1 mediated by Ubcd1 (an E2 ubiquitin-conjugating enzyme) and activation of Dronc [49,50]. Like in spermatid individualization, the apoptotic machinery is also used in a spatially restricted way to destroy only parts of a cell. The precise regulatory mechanisms to achieve this local activation of caspases are still poorly understood. Diap1, Dronc and Dark (Apaf1 homologue) have also a non-apoptotic role during border cell migration in the *Drosophila* ovary [51].

Another example of the non-apoptotic use of caspases is the specification of neural precursor cells in *Drosophila* [Kuranaga and Miura, 2007]. The *Drosophila* IKK-related kinase (DmIKKε) was identified as a new regulator of Diap1 protein degradation [52]. DmIKKε directly binds and phosphorylates Diap1, promoting Diap1 degradation. Loss of function of DmIKKε by RNAi led to the upregulation of Diap1 protein levels, and resulted in the formation of extra macrochaetae due to reduced caspase activity. However, cell death during development was not affected by the loss of DmIKKε function [52]. Regulation of Diap1 by DmIKKε is also important for organization of the actin cytoskeleton and changes in cell morphology

[53]. Collectively, these studies raise the interesting question of how the potentially lethal activity of apoptotic proteins is restricted to specific compartments within a cell.

Conclusions

Although in a few select cases the signaling pathways regulating cell death and their intersection with the core apoptotic program are known in considerable detail, in general we still do not know much about how a particular cell chooses between life and death during normal development. Given the terminal nature of cell death and the potentially catastrophic consequences of mistakes, it should not come as a surprise that many distinct layers of control are used to tightly regulate the activity of caspases. This includes transcriptional regulation of caspases, activation of caspases by apoptosome formation, degradation of pro-caspases (such as Dronc) by IAPs and the ubiquitin-proteasome system, activation of caspases through inhibition of IAPs by Reaper-family proteins, regulation of core cell death proteins by phosphorylation, micro-RNAs, and by changing their subcellular localization. Furthermore, it has become increasingly clear that apoptotic cells can actively communicate with their cellular environment, and not just to attract phagocytes, but also to stimulate cell proliferation and tissue regeneration. Finally, core components of the cell death program, including apoptotic effector caspases, were found to have non-apoptotic roles in cellular remodeling, differentiation and cell specification. With each advance many more new questions have emerged that will undoubtedly keep the field busy for years to come.

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Figure 1.

The core apoptotic pathways in *C. elegans*, *Drosophila* and mammals. The functional homologues between species are represented with boxes with the same background color. For the sake of simplicity, we did not include the pathways leading to the activation of caspase-2 and caspase-8 (extrinsic pathway).