Chapter 1: Developmentally Regulated Programmed Cell Death in *C. elegans* and *Drosophila*

Death is essential to life. Programmed cell death, the removal of certain cells, allows multicellular organisms the ability to sculpt developing tissue or protect themselves from disease. The most studied form of programmed cell death is apoptosis, a highly conserved, intrinsic process characterized by a family of proteases called caspases. Initially formed as inactive zymogens, caspases themselves undergo several proteolytic cleavages to form active homomeric enzymes. Once activated, caspases induce gross morphological changes in the cell, such as DNA fragmentation and membrane blebbing.

Death in the nematode *Caenorhabditis elegans*

The initial studies in the nematode *Caenorhabditis elegans* showed that apoptosis is a carefully regulated genetic process. Genetic screens isolated a class of mutants, the so-called *ced* genes for CEll Death abnormality, that are important for the death of 131 somatic cell during *C. elegans* development (Ellis and Horvitz, 1986). Follow-up screens identified a total of 14 loci that fall into two broad functional catergories: cell killing or corpse removal. Epistatis analysis of the *ced* mutants revealed a basic genetic cascade wherein CED-3, CED-4, and EGL-1 are necessary for cell death, and CED-9 inhibits the function of CED-4. Characterization of the *C. elegans ced* genes showed the conserved

nature of the apoptotic machinery and laid the foundation for subsequent studies in other invertebrates and mammals (reviewed in Aravind et al., 2001; Danial and Korsmeyer, 2004). Part of a broad family, CED-3 encodes a caspase with its proteolytic cysteine residue and a long regulatory prodomain (Yuan et al, 1993). CED-4 promotes apoptosis by facilitating the oligomerization and activation of CED-3 and was later shown to be homologous to the mammalian Apaf-1 (Zou, et al, 1997; Yang et al., 1998). Loss of the anti-apoptotic *ced-9* causes ectopic cell death and sterility; *ced-9* is structurally similar to the proto-oncogene Bcl2 (Hengartner et al., 1992; Hengartner and Horvitz, 1994). EGL-1 is a BH3-domain protein, a subclass of the Bcl2 family, that promotes cell death by directly inhibiting the protective CED-9 (Conradt and Horvitz, 1998). The developmental regulation of cell death is critical and the invertebrates *C. elegans* and *Drosophila* provide important examples for understanding this regulation.

Cell death in *C. elegans* is transcriptionally activated

Two main points emerge from experiments in *C. elegans*. First, it is now clear that cells are primed to die. The apoptotic machinery, specifically CED-3 and CED-4, is present in most, if not all, cells, though these proteins are inhibited by CED-9. Loss of CED-9 relieves the inhibition of the death machinery and causes death in cells not normally slated to die (Hengartner et al., 1992). In fact, more cell death would be observed in *ced-9* mutants if it were not for the maternal deposit of CED-9 to the progeny. If all cells have the death machinery present and have the ability to kill themselves rapidly, these

observations underscore the need for the careful regulation of CED-9 to maintain viability.

Secondly, studies show that death in *C. elegans* is transcriptionally activated by the upregulation of the CED-9 inhibitor EGL-1. The nervous system of *C. elegans* is a sexually dimorphic structure; for example, the HSN neurons die in embryonic males, but are essential for hermaphrodite egg laying. The sex determining factor TRA-1A is responsible for this dimorphism by altering the transcription of several genes. One target of TRA-1A is the pro-apoptotic *egl-1* gene. TRA-1A inhibits the transcription of *egl-1* in hermaphroditic HSNs, and therefore promotes their survival, while in embryonic male HSNs, TRA-1A is absent, causing the transcription of *egl-1* and the eventual activation of the CED-3 caspase (Conradt and Horvitz, 1999). Such transcriptional regulation of egl-1 and cell death has been also shown for the NSM sister cells (Thellmann et al., 2003).

The *Drosophila* model of apoptosis

Studies on the fruit fly *Drosophila melanogaster* have shown that only certain aspects of cell death are conserved. First of all, many of the genes involved in apoptosis are similar in *C. elegans* and *Drosophila* (reviewed in Vernooy et al., 2000). Though not all are involved in mediating apoptosis, the fly has seven caspases that can be grouped into two categories based on the presence of a regulator prodomain. The three upstream or apical caspases, *Dronc*, *Dream*, and *Dredd*, have long prodomains, and the prodomain of *Dronc* has a CARD domain also found in CED-3 and CED-4. The effector or downstream

caspases, *DrICE, dcp-1*, *decay*, and *damm*, have short prodomains, and are the real mediators of apoptosis. Effector caspases are proteolytically activated by the apical group, which adds a layer of regulation for the cell to maintain viability. Dark is functionally and structurally similar to CED-4; it is bound to the mitochondria and facilitates the activation and processing of the apical caspase Dronc (Rodriguez et al, 1999). The conservation of structure and function with CED-4 and Dark represents the most significant similarity between the *C. elegans* and *Drosophila* apoptotic machinery.

Here the similarity ends, and flies and worms have different mechanisms for the inhibition and activation of caspases. Because *Drosophila* has both the apical and effector caspase groups, caspases can assume different functions and show tissue specificity. The caspase Dredd for example is involved in the innate immune response (Elrod-Erickson et al., 2000). Secondly, in worms, the model of caspase inhibition is in part a consequence of CED-9 sequestering CED-4 to the mitochondria; in contrast, the *Drosophila* DIAP1 protein directly inhibits the processing of the Dronc zymogen and the active form of DrICE (Chai, et al., 2003; Yan et al., 2004). Finally, the activation of programmed cell death in *C. elegans* is mediated by the transcriptional upregulation of *egl-1*. Flies encode their own Bcl2 family members, *Debcl* and *Buffy*, which have been implicated in cell death, though the mechanism of their action is still unclear (Colussi et al., 2000; Quinn et al., 2003). To activate caspase-mediated cell death, *Drosophila* uses the small genes *head involution defective* (*hid*), *grim*, and *reaper*. HID, Grim, and Reaper have a small N-terminal motif that binds to DIAP1 and relieves the caspase inhibition (Wu et al., 2001). The mammalian proteins Smac/Diablo play a functionally

similar role to HID, Grim, Reaper, though there is little sequence similarity between the proteins (Du, et al., 2000; Verhagen et al., 2000). Regulation of *hid*, *grim*, and *reaper* occurs by various mechanisms. The microRNA *bantam* can control the translation of HID, though the developmental context of this regulation has yet to be found (Brennecke et al., 2003). The homeobox gene *Deformed* upregulates the transcription of *reaper* to cause death in the head segments in the developing embryo (Lohmann et al., 2002). Though discussed in more detail later, the EGF receptor pathway has been shown to attenuate HID activity both transcriptionally and post-transcriptionally (Kurada and White, 1998; Bergmann et al., 1998).

Programmed cell death in *Drosophila* development

The regulation of the death machinery in fly development has not been fully characterized. Cell death is important for the formation of several adult tissues as well as the removal of larval tissue during the pupal stage. Investigations of cell death during *Drosophila* development follow two approaches. Using a forward genetics approach, observation of developing tissues and characterization of mutants has yielded a class of genetic mutants. For example, *hid* was originally isolated as a mutant that displayed defects in male terminalia rotation and embryonic head involution (Abbott and Lengyel, 1991). Characterization of the long-standing mutants *roughest* (*rst*) and *echinus* (*ec*) showed that apoptosis is a natural part of the development of the adult retina (Wolff and Ready, 1991). Observations of wings from recently eclosed animals revealed that cell death was necessary for proper wing formation (Kimura et al, 2004).

The second approach involves the characterization of genes known to activate apoptosis by looking at gene expression patterns or, if available, the mutant phenotypes. The death activator *Dark* was cloned by its homology to CED-4 and then shown to be important for wing and scutellar bristle formation (Rodriguez et al., 1999). Gene expression patterns for *grim* revealed that the gene is transcribed just prior to the onset of cell death in embryonic tissues (Chen et al., 1996).

Steroid-induced cell death

Developmental cell death is particularly striking during *Drosophila* metamorphosis. During the pupal stage the tissues from the larval stage must make room for the proliferating cells that will make up the adult fly. At this time, the steroid hormone ecdysone is released in several bursts to facilitate the formation of the puparium and the destruction of the larval tissue. EcR and USP comprise the hormone receptor, which then relays a signal through βFTZ-F1, BR-C, E74, and E93 to activate transcription (Jiang et al., 2000). Simultaneous to the destruction of the larval salivary gland and midgut, *E93* induces the transcription of the pro-apoptotic genes *Dronc*, *hid*, *reaper*, and *dArk* (Lee et al., 2000). Other caspases besides *Dronc* must be involved as the caspase inhibitor P35 can delay pupal cell death and *Dronc* is P35 insensitive (Jiang et al., 1997).

One curious observation of steroid-triggered cell death is that dying tissues display some non-apoptotic morphologies. Autophagic cell death is a less characterized mode of cell

death distinguished by large cytoplasmic vacuoles. Dying salivary glands display the autophagic vacuoles as well as markers for active caspases, like altered cytoskeleton proteins and DNA fragmentation (Martin and Baehrecke, 2004). Though the role of autophagic cell death in dying pupal tissue is not understood, caspases and their activators HID, Grim, and Reaper are essential.

Signaling and pattern formation in the eye

The developing adult eye presents a juxtaposition of various cellular signals that integrate cell differentiation, apoptosis, and cell adhesion. The adult eye is composed of approximately 750 repeating units called ommatidia. Three cell types comprise each ommatidium: the photoreceptors, the cone cells, and primary pigment cells. Interspacing the ommatidia are a collection of cells known as interommatidial cells (IOCs) and bristles. Adult eye formation begans in the third instar larval stage when the morphogenetic furrow sweeps anteriorly across the eye imaginal disc. The morphogenetic furrow synchronizes the cell cycle of the epithelial cells just prior to it, and then initiates the differentiation of those epithelial cells. The photoreceptors are the first cluster of cells to differentiate and can be visualized during the larval stage. The remaining cells differentiate during the pupal stage of development, with the cone cells first, followed by the primary pigment cells, then finally the IOCs.

In part, pattern formation of the fly eye is dependent on caspases and cell death. An understanding of the importance of cell death in late eye development began with the

characterization of the *echinus* and *roughest* mutants. These two mutants display rough, non-ordered eyes due to the lack of apoptosis late in cell differentiation (Wolff and Ready, 1991). It was shown that after IOCs differentiate, a few unselected cells die in the normal course of development to complete the ordered ommatidia array. Later studies showed that pupal eye death can be blocked by overexpression of P35 or a mutation in *hid* (Hay et al., 1994; Yu et al., 2002). Mutants of *morgue*, a gene involved in ubiquitin-mediated protein degradation, show altered levels of DIAP1 and excess IOCs (Hays et al., 2002; Wing et al., 2002).

Experiments have also implicated several signaling pathways in late eye development. Most notably, a dominant-negative version of the EGF receptor (*DER*) disrupts normal differentiation of all eye cells including the IOCs, which underscores its necessity throughout eye development (Freeman, 1996). Likewise, overexpression of *Argos*, a natural inhibitor of the EGF receptor, mimics the phenotypes of dominant-negative *DER* (Sawamoto et al., 1998). Recent work also revealed the role of the transcription factor *lozenge* during late eye development. Loss of *lozenge* in late eye development decreases the amount of cell death, but not the differentiation of IOCs (Wildonger et al., 2005). Interestingly, *lozenge* was also shown to upregulate the transcription of *Argos*, providing a mechanism for control of retinal cell death by lozenge (Wildonger et al., 2005).

The other cellular factor involved in pupal eye apoptosis is cell adhesion. *roughest*, a mutant identified by a lack of cell death specifically in the eye, was later cloned as a novel immunoglobulin gene (Ramos et al., 1993). Antibodies revealed that proper

localization of Roughest to the cell borders between primary pigment cells and IOCs was necessary for apoptosis and was dependent on the cell adhesion molecule DE-cadherin (Reiter et al., 1996; Grzeschik and Knust, 2005).

Clues are emerging as to how the retinal cells sense and integrate signals emitted during cell adhesion, cell differentiation, and cell death. The EGF receptor DER and the downstream component Ras have been shown to suppress the activity of HID by modulating its transcription and phosphorylation state (Kurada and White, 1998; Bergmann et al., 1998). These results suggest that DER/Ras provide a cell survival signal by inactivating HID and, therefore, the caspases. The Notch signaling pathway has been proposed either to promote IOC cell death or to inhibit cell survival (Miller and Cagan, 1998). One mechanism for Notch activity is through Roughest. Mutants for *Notch* affect the localization of Roughest in the pupal eye, which, as mentioned before, is necessary for retinal apoptosis (Gorski et al., 2000).

Despite all these gains, important questions remain about cell death in the pupal eye. It still remains unclear how the cell senses proper cell adhesion. Why is proper localization of Roughest to the border between IOCs and primary pigment cells critical for cell survival? Another remaining question revolves around the mutant *echinus*. *echinus* was originally isolated by Bridges in 1916 because of its rough eye phenotype, and subsequently it was used as an X-linked marker (Lindsley and Zimm, 1992). Later studies show that mutants for *echinus* disrupt pupal eye cell death, much like *roughest* (Wolff and Ready, 1991). But since then *echinus* has remained uncharacterized. Finally,

it is unknown what signals induce apparently equal interommatidial cells to either survive or commit cell suicide. It could be that improper or insufficient Roughest localization causes the activation of Lozenge, which in turn inhibits the EGFR cell survival signal, resulting in cell death. Another outstanding question that remains in *Drosophila* is how active caspases can be present in border cell migration in oogenesis and in sperm individualization, yet not induce cell death (Geisbrecht and Montell, 2004; Huh et al., 2004). Finally, it has been noticed that HID is present in cells of the primordial optic lobe that do not die (Grether et al., 1995). It is yet to be determined what prevents HID from activating cell death in this context.

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