Topic Introduction

Analysis of Apoptosis in *Caenorhabditis elegans*

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The nematode worm *Caenorhabditis elegans* has provided researchers with a wealth of information on the molecular mechanisms controlling programmed cell death (apoptosis). Its genetic tractability, optical clarity, and relatively short lifespan are key advantages for rapid assessment of apoptosis in vivo. The use of forward and reverse genetics methodology, coupled with in vivo imaging, has provided deep insights into how a multicellular organism orchestrates the self-destruction of specific cells during development and in response to exogenous stresses. Strains of *C. elegans* carrying mutations in the core elements of the apoptotic pathway, or in tissue-specific regulators of apoptosis, can be used for genetic analyses to reveal conserved mechanisms by which apoptosis is regulated in the somatic and reproductive (germline) tissue. Here we present an introduction to the study of apoptosis in *C. elegans*, including current techniques for visualization, analysis, and screening.

BACKGROUND

Somatic Apoptosis

During embryonic development, 113 cells die by apoptosis in the *Caenorhabditis elegans* hermaphrodite, and an additional 18 cells undergo apoptosis during the larval stages (Sulston and Horvitz 1981; Sulston et al. 1983). Apoptotic corpses persist for ~40 min and are easily distinguished from healthy cells by their refractile "button-like" appearance under differential interference contrast (DIC) optics—the gold standard for quantification of apoptosis in *C. elegans*.

Forward genetics screens have identified four genes that comprise the core apoptosis pathway in *C. elegans* (Fig. 1). This pathway is required for the execution of almost all cells fated to die during development and in response to stress (Ellis and Horvitz 1986; Hengartner et al. 1992; Yuan and Horvitz 1992; Yuan et al. 1993; Conradt and Horvitz 1998; Gumienny et al. 1999; Gartner et al. 2000; Horvitz 2003). The cell death abnormal gene 3, *ced-3*, encodes a proteolytic caspase protein that is activated by the protein product of the *ced-4* gene, the worm homolog of mammalian apoptotic protease activation factor 1 (Apaf-1) (Yuan and Horvitz 1992; Yuan et al. 1993). The *ced-9* gene encodes a protein that is homologous to the anti-apoptotic B-cell lymphoma 2 (Bcl-2) family of proteins. Ablation of *ced-9* results in massive levels of ectopic apoptosis that are completely suppressed by loss-of-function mutations in *ced-4* or *ced-3*, which was the first genetic evidence that *ced-9*/Bcl-2 functions upstream of *ced-4*/Apaf-1 to prevent activation of the CED-3 caspase (Hengartner et al. 1992; Hengartner and Horvitz 1994b). Upstream of *ced-9* is the egg laying defective 1 (*egl-1*) gene, which encodes a proapoptotic BH3-only protein that antagonizes the CED-9 protein (Conradt and Horvitz 1998).

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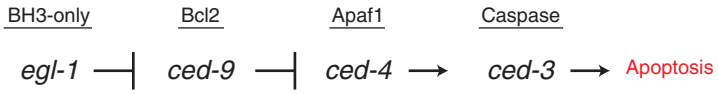

FIGURE 1. The core apoptosis pathway of *C. elegans*. Mammalian counterparts are indicated *above* the worm gene names.

The invariant lineage of *C. elegans* has been a major advantage for identifying different regulators of the core apoptosis pathway (see the review by Conradt and Xue [2005]). Activation generally involves increased transcription of *egl-1* by tissue-specific transcription factors. (One notable exception is the male linker cell, which undergoes cell death by a mechanism independent of *egl-1* and the core apoptosis pathway [Abraham et al. 2007; Blum et al. 2012].) Over-expression of apoptosis genes using heat-inducible or lineage-specific promoters can activate (or inhibit) apoptosis in various mutant backgrounds to help establish epistatic relationships (see, for example, Hengartner and Horvitz 1994b; Shaham and Horvitz 1996a,b; Conradt and Horvitz 1998). The ability to perform genetic epistasis experiments with relative ease is one of the key advantages of using *C. elegans* to delineate cell death signaling pathways.

Once committed to apoptosis, the fated cells are recognized by exposed phosphatidylserine (PS) on their surface (the "eat-me" signal), then swiftly engulfed by healthy neighboring cells (Ellis et al. 1991; Grimsley and Ravichandran 2003; Kinchen and Ravichandran 2007). Critical to engulfment is the CED-1 protein, which recognizes corpses following PS exposure; CED-1 can be fused with fluorescent tags and used as an in vivo marker of apoptosis (Zhou et al. 2001; Kinchen et al. 2008). Loss-of-function mutations in engulfment genes such as *ced-1* cause cell corpses to persist, making detection of subtle changes in apoptosis more sensitive when counting corpses in embryos at various stages of development. Because the somatic lineage is invariant, and a number of apoptotic deaths during development occur in the anterior pharynx, it is easy to detect extra "undead" cells in this organ using DIC optics (Fig. 2). (For a comprehensive description of the method for counting cells in the pharynx, we refer readers to the protocol by Schwartz [2007].)

Germline Apoptosis

Physiological Apoptosis

The germline of *C. elegans* is comprised of two symmetric bilateral tubes that contain a population of mitotically proliferating stem cells at their distal ends. As these cells continue to proliferate, they move in a proximal direction and enter the stages of meiosis I. As meiotic cells exit the pachytene stage (around the bends of the gonad arms) they become competent to undergo apoptosis, and it has been estimated that ~50% of germ cells undergo physiological apoptosis (Gumienny et al. 1999). Similar to developmental cell death in embryos and larvae, apoptotic germ cell corpses display a refractile "button-like" morphology that makes them easy to distinguish by DIC optics (Fig. 3). Mutations in engulfment genes, such as *ced-1*/CD91 or *ced-6*/GULP, cause germ cell corpses to persist because they cannot be engulfed by the surrounding gonadal sheath cells (Gumienny et al. 1999). While the core execution genes *ced-4* and *ced-3* are required for physiological germ cell apoptosis, *egl-1* is dispensable, and a gain-of-function mutation in *ced-9* which suppresses developmental apoptosis in the soma and stress-induced apoptosis in the germline does not affect physiological germ cell apoptosis (Hengartner

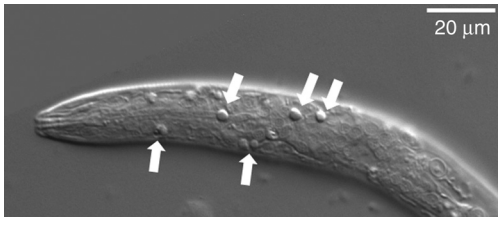

FIGURE 2. Pharyngeal apoptosis in *C. elegans*. The distinct "button-like" morphology is seen in the anterior pharynx at the L1 stage of development. Several corpses arising from developmental apoptosis persist in the head region of animals carrying a mutation in the engulfment gene *ced-5*. Worm viewed by DIC optics using a 63× oil immersion lens.

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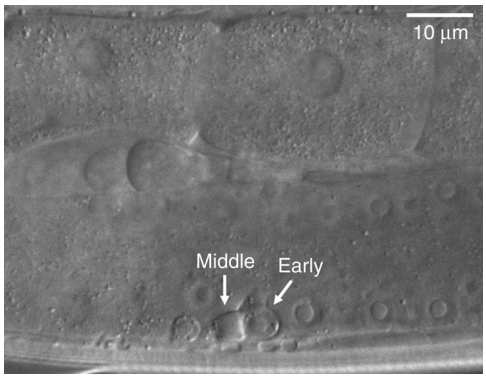

FIGURE 3. Germline apoptosis in *C. elegans*. Corpses appear as "buttons" after they mature and cellularize out of the germline syncytium. Different stages of apoptosis are shown, as the corpse begins to cellularize (early) and form the distinct "button-like" morphology (middle/late) before undergoing engulfment by the surrounding gonadal sheath cells. The process from budding to engulfment takes ~40 min. Germline viewed by DIC optics using a 63× oil immersion lens.

and Horvitz 1994a; Gumienny et al. 1999; Gartner et al. 2000). Thus, there are some interesting differences in how the core cell death pathway regulates apoptosis in the germline versus the soma.

Physiological germ cell apoptosis requires *let-60*/Ras signaling, but because this pathway is also required for germ cells to exit the pachytene stage of meiosis I (where they become competent to undergo apoptosis), it has been unclear whether Ras signaling imparts a direct or indirect effect on germline apoptosis (Gumienny et al. 1999). Recent evidence suggests a more direct role for Ras signaling in promoting germ cell apoptosis, possibly through a combination of *C. elegans* p53-like (*cep-1*)-dependent and -independent mechanisms (Kritikou et al. 2006; Rutkowski et al. 2011; Perrin et al. 2013). Ras pathway activity can be monitored by staining dissected germlines with commercial antibodies to phosphorylated mammalian ERK, which cross-reacts with *C. elegans* phospho-MPK-1 (Arur et al. 2009; Rutkowski et al. 2011; Perrin et al. 2013) (see Protocol: **Immunostaining for Markers of Apoptosis in the *Caenorhabditis elegans* Germline** [Lant and Derry 2014a]). This method provides a reliable readout for assessing Ras activity in the germline, which can be examined in various mutant backgrounds and under different conditions.

Stress-Induced Apoptosis

Germ cells of the *C. elegans* hermaphrodite also undergo apoptosis in response to a variety of environmental stresses, such as DNA damaging agents. (Germ cells of males, however, do not undergo physiological or stress-induced apoptosis.) Stress-induced germ cell apoptosis requires a conserved DNA damage checkpoint and the *cep-1* gene, which is not required for physiological germ cell apoptosis (Gartner et al. 2000; Derry et al. 2001; Schumacher et al. 2001; Stergiou and Hengartner 2004; Gartner et al. 2008). Unlike physiological germ cell apoptosis, stress-induced germ cell apoptosis requires *egl-1,* and a gain-of-function allele in *ced-9* suppresses apoptosis in response to DNA damaging agents (Gartner et al. 2000). In response to DNA damaging agents, such as ionizing radiation (IR), alkylating agents or ultraviolet (UV) irradiation, endogenous CEP-1 protein becomes phosphorylated and activates the transcription of *egl-1* (Hofmann et al. 2002; Quevedo et al. 2007; Gao et al. 2008). CEP-1 activity is negatively regulated by the Akt/PKB kinase AKT-1, the GLD-1 RNA-binding protein, and the E3 ubiquitin ligase SCF^{FSN-1}, which is evident by elevated levels of phosphorylated CEP-1 and *egl-1* transcript in strains carrying loss-of-function mutations in these genes after treatment with DNA damaging agents (Schumacher et al. 2005; Quevedo et al. 2007; Gao et al. 2008). The human equivalent of the SCF^{FSN-1} E3 ubiquitin ligase complex, SCF^{FBXO45}, negatively regulates the p53 family member p73, revealing conservation from worm to human (Peschiaroli et al. 2009). The relative level and subcellular localization of endogenous CEP-1 (as well as core apoptosis proteins) can be monitored by staining dissected germlines with antibodies (see Fig. 1 in Protocol: **Immunostaining for Markers of Apoptosis in the *Caenorhabditis elegans* Germline** [Lant and Derry 2014a]), and CEP-1 phosphorylation status can be analyzed by western blotting (Quevedo et al. 2007; Gao et al. 2008; Sendoel et al. 2010). For an accurate and reproducible estimate of CEP-1 transcriptional activity, *egl-1* transcript levels can be measured by quantitative real-time PCR (Hofmann et al. 2002).

GENETIC ANALYSIS OF APOPTOSIS IN *C. ELEGANS*

C. elegans is an ideal organism for analyzing the genetic control of apoptosis and establishing order in relevant signaling pathways. A vast array of strains is available and encompasses mutations in genes required at all stages of the apoptotic process, including lineage-specific regulators, core pathway components, and corpse engulfment genes. Strains can be ordered by mail from the *Caenorhabditis* Genetics Center (http://www.cbs.umn.edu/CGC/) for a small fee. When analyzing mutants that have abnormally high numbers of refractile corpses, it is important to confirm that these are indeed apoptotic corpses by generating double-mutant strains containing loss-of-function mutations in the core executioner genes *ced-4* or *ced-3*. Because these genes are required for all apoptotic cell death in *C. elegans*, the appearance of abnormal numbers of corpses should be suppressed by *ced-3* and *ced-4* loss-of-function mutations. Likewise, mutants that cause reduced numbers of apoptotic corpses should be crossed into a strain carrying a loss-of-function mutation in the anti-apoptotic gene *ced-9*. If apoptosis is restored by ablation of *ced-9*, then it can be concluded that the core apoptotic machinery is functional in these mutants. Alternatively, ablation of these genes by RNAi can be performed to help establish epistatic relationships in mutants with abnormal numbers of apoptotic corpses (see Protocol: **Induction of Germline Apoptosis in *Caenorhabditis elegans*** [Lant and Derry 2014b]). However, because the penetrance of RNAi can be variable, we recommend that epistatic relationships be confirmed using genetic mutants. Similar to immunostaining for CEP-1 protein, the germlines of mutants with altered levels of apoptosis (physiological or stress-induced) can be stained with antibodies to core apoptosis proteins, such as CED-9 and CED-4, to determine if their levels or localization are altered (Greiss et al. 2008; Perrin et al. 2013; Pourkarimi et al. 2012). For example, using a combination of genetic epistasis and immunocytochemistry, the DAF-2 insulin-like receptor was shown to promote IR-induced germ cell apoptosis by attenuating Ras signaling without detectably affecting the levels or localization of core apoptosis proteins (Perrin et al. 2013). The combination of genetics and cell biology reagents provides a basic toolkit for defining the mechanisms by which various proteins control apoptosis.

Another advantage of *C. elegans* is the availability of tissue-specific promoters to determine the site of focus for apoptosis regulators. For example, physiological and DNA damage-induced germline apoptosis is under regulatory control of both autonomous and nonautonomous signaling molecules (Ito et al. 2010; Sendoel et al. 2010; Li et al. 2012). In addition, *C. elegans* is ideally suited for performing high-throughput reverse genetics screens for apoptosis regulators. Using liquid RNAi cultures in a 96-well plate format, along with the vital dye acridine orange, the entire genome of the nematode can be screened for apoptosis genes with unprecedented efficiency (Lettre et al. 2004) (see Protocol: **High-Throughput RNAi Screening for Germline Apoptosis Genes in *Caenorhabditis elegans*** [Lant and Derry 2014c]).

SUGGESTED TECHNIQUES

We have prepared a series of protocols which cover the basics of assessing apoptosis in *C. elegans*, from basic techniques for visualizing corpses in embryos and the adult germline to the use of vital dyes, fluorescent strains and immunostaining for visualizing the localization and levels of endogenous apoptosis proteins (see Protocol: **Induction of Germline Apoptosis in *Caenorhabditis elegans*** [Lant and Derry 2014b]; Protocol: **Visualizing Apoptosis in Embryos and the Germline of *Caenorhabditis elegans*** [Lant and Derry 2014d]; Protocol: **Fluorescent Visualization of Germline Apoptosis in Living *Caenorhabditis elegans*** (Lant and Derry 2014e); Protocol: **High-Throughput RNAi Screening for Germline Apoptosis Genes in *Caenorhabditis elegans*** [Lant and Derry 2014c]; Protocol: **Immunostaining for Markers of Apoptosis in the *Caenorhabditis elegans* Germline** [Lant and Derry 2014a]). The use of RNAi, either for individual or large groups of genes, enables assessment of apoptotic phenotypes within a few days by simply cultivating worms on bacterial strains expressing double-stranded RNA targeting the gene(s) of interest. Taken as a whole, these protocols allow a

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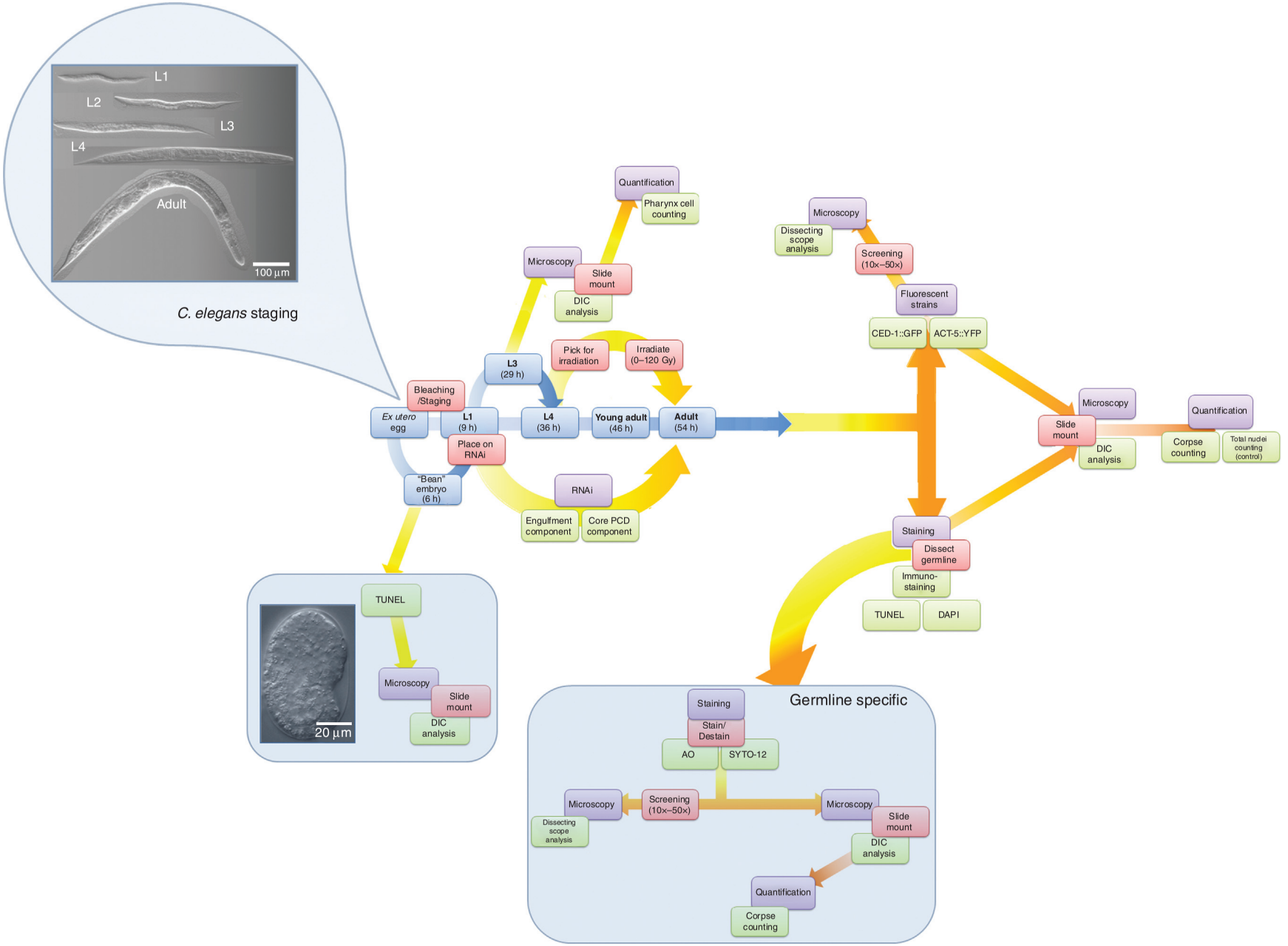

FIGURE 4. An overview of methods used to assess apoptosis in the soma and germline of *C. elegans*. As indicated, some procedures are stage-specific, or only valid in germline analysis.

novice *C. elegans* researcher to quickly develop expertise in assessing apoptosis. They also facilitate methods for deeper analysis, such as high-resolution imaging, genetic epistasis analysis, and genome-wide modifier screens. A flowchart guide to the analysis of apoptosis in *C. elegans* (Fig. 4) places the skills and techniques outlined in our protocols in chronological order according to developmental stages of the worm.

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