Studies of Programmed Cell Death
in the Nematode *Caenorhabditis elegans*

by
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Abstract

Programmed cell death is an evolutionarily conserved process that plays critical roles in normal animal development and has been extensively studied in *C. elegans*. During programmed cell death, caspases are activated in the dying cell. The cell corpse is engulfed by a neighboring cell and degraded. Almost all cell deaths in *C. elegans* are "suicides"—they are caspase-dependent and apparently cell-autonomous, and do not require engulfment.

During development of the *C. elegans* male, the cells B.alapaav and B.arapaav are generated during the late third-larval stage. During the early fourth-larval stage one of these cells undergoes programmed cell death, and the other survives. These two cells form an equivalence group; the decision of which cell dies and which survives is stochastic. The cell that dies is engulfed by the neighboring cell P12.pa and was speculated to be an engulfment-dependent cell "murder" or an "induced suicide." I have discovered that B.al/rapaav instead represents an "assisted suicide" that requires both the core apoptosis pathway and the engulfment pathway. *egl-1* and *ced-3* are expressed in the dying or undead cell in wild-type and engulfment-defective animals, and these genes are required for the B.al/rapaav cell death. In engulfment mutants the B.al/rapaav death process fails at a point after caspase activation, suggesting that the core cell-death pathway is necessary but not sufficient for this cell death.

Previous genetic screens have not been designed to systematically identify essential genes with a role in cell death. Most somatic cell deaths in *C. elegans* occur during early development, but several male-specific cell deaths occur during the fourth larval stage. These late cell deaths provide an opportunity to examine essential genes for a role in programmed cell death, as RNAi treatment after hatching can eliminate gene function before these deaths occur but after embryogenesis. I performed an RNAi screen for 1,132 essential genes and assayed the effect on Rn.aap cell survival. I analyzed candidate genes for non-specific effects, such as affecting the Rn cell lineage rather than cell death processes, to find twenty-five essential genes that might have a role in the Rn.aap cell death.

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This is the part of my thesis that I have fantasized about the most! Graduate school has had many ups and downs for me, and I am so grateful for the people who have helped along the way, both scientifically and personally.

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Figure 1 Young Holly looks at pond water under the microscope.

Finally, thank you to the many *C. elegans* I have known. I will never forget the first cell I saw divide, or the first time I finally met B.alapaav and B.arapaav.

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Chapter 1

Introduction

Programmed cell death and apoptosis

1. Apoptosis is defined by morphological characteristics

The observation that cells could be lost was first made in 1842 (Vogt). In the nineteenth century, the existence of cell death was "discovered" at least six more times by researchers who independently observed it (Clarke and Clarke, 1996). These early scientists observed that cells died in many types of organisms; during metamorphosis, normal development, and in the adult; and with a variety of morphologies, depending on the cause of the cell death (reviewed in Glücksmann, 1951). Developmental cell deaths share a common set of ultrastructural alterations: the chromatin condenses, the nucleus and cytoplasm shrink, and the membrane extends protrusions that can bud off from the rest of the cell (Glücksmann, 1951; Kerr *et al.*, 1972; Wyllie *et al.*, 1980). Meanwhile, the organelles remain intact. This morphology is in contrast to necrosis, pathological cell death caused by injury, in which organelles swell and the membrane integrity is compromised (Wyllie *et al.*, 1980). The developmental cell deaths were called "cellular degenerations" and "shrinkage necrosis" (Glücksmann, 1951; Kerr, 1971). In 1964 they began to be referred to as programmed cell deaths, acknowledging that they occurred in reproducible times and places, and therefore were likely to be genetically controlled (Lockshin and Williams 1964). Later it became apparent that these developmental cell deaths are of fundamental biological importance as a balance to cell proliferation, and they were termed "apoptosis" (Greek for "falling off") to distinguish them from necrotic cell deaths due to injury (Kerr *et al.*, 1972). Kerr *et*

al. further speculated that decreased apoptosis might contribute to cancer, and that induction of apoptosis could promote tumor regression, ideas that are now widely accepted, as will be described further below.

2. Dying cells are engulfed and removed

Early after the discovery of dying cells, it was observed that they were rapidly engulfed by other cells (Clarke and Clarke, 1996; Metschnikoff, 1883) Apoptotic bodies, small vesicles that bud off of dying cells, were visible inside of living cells. It was a subject of debate whether the engulfing cells, phagocytes, caused the death of the dying cells or if they played a secondary role, functioning only to clean up the cell corpses; however it was observed that cell death in regressing muscle cells during frog metamorphosis seemed to be initiated before the arrival of phagocytes, and thus phagocytosis was not required for apoptosis to occur (Clarke and Clarke, 1996). In addition, dying cells that were shed instead of being engulfed still displayed signs of degradation (Glücksmann, 1951). With the discovery of mutants deficient in engulfment in *C. elegans,* it became clear that cell death can proceed independently of phagocytosis (Hedgecock *et al.,* 1983). However, the idea that phagocytosis does not contribute to cell death has been challenged more recently, which will be discussed below and in the rest of this thesis.

3. Apoptosis plays roles in development and homeostasis

Apoptosis plays fundamental roles in biology, both during development and throughout the life of an organism. During development, cell death removes unnecessary cells, such as Müllerian ducts in males and Wolffian ducts in females to

promote sexual differentiation (Ortiz, 1945) or cells between the toes to prevent webbed feet in some animals (Mori *et al.,* 1995; Saunders, 1966). In some cases, such as neurons, cells are created in excess and those that do not make the correct connections or are unneeded undergo cell death (Hamburger and Levi-Montalcini, 1949; Hamburger *et al.,* 1981). Apoptosis also promotes morphogenesis, such as invagination in the neural plate or the formation of lumen in the colon once the inner cells die (Glücksmann, 1951). During metamorphosis, larval cells that are no longer needed, such as a tadpole's tail, regress through programmed cell death (Bataillon, 1891; Eberth, 1866).

Apoptosis is also required for homeostasis and to protect the organism from potentially harmful cells. Proliferation of cells by mitosis must be balanced by death of cells to maintain cell number (Kerr *et al.*, 1972). Cells that become damaged by mutations can be induced to undergo apoptosis by expression of the tumor suppressor gene p53, which prevents them from doing harm to the organism (Kastan *et al.,* 1991; Yonish-Rouach *et al.*, 1991). Immune cells that are autoreactive are induced to die by apoptosis to prevent autoimmune disease (Jenkinson *et al.,* 1989; Surh and Sprent, 1994). In these ways, apoptosis contributes both to the proper development as well as the healthy survival of a multicellular organism.

4. Apoptosis plays roles in disease

Consistent with the critical role apoptosis plays in development and homeostasis, dysregulation of apoptosis is associated with many diseases (Favaloro *et al.*, 2012; Thompson, 1995). Increased programmed cell death has been implicated in degenerative diseases such as retinal dystrophy and damage after

ischemia-reperfusion. Rats with inherited retinal dystrophy have death of photoreceptors with DNA degradation consistent with this death occurring through apoptosis (Tso *et al.*, 1994) and increased expression of the pro-apoptotic protein Bax (Katai *et al.*, 2006). Ischemia such as stroke or myocardial infarction causes cell death by both apoptosis and necrosis (Hutchins and Barger, 1998). Loss of cell death genes, such as caspase-3 in neurons, both *in vitro* and *in vivo* (Le *et al.*, 2002) and Fas in heart cells (Lee *et al.*, 2003) have been shown to be protective during ischemia. Conversely, overexpression of anti-apoptotic genes can reduce infarct size after ischemia-reperfusion (Chen *et al.*, 2001; Chua *et al.*, 2007).

Mutations that inhibit programmed cell death allow over-proliferation and are one of the hallmarks of cancer (Fulda, 2009; Hanahan and Weinberg, 2000). The anti-apoptotic gene *bcl-2* was named for its role in causing B-cell lymphoma when a translocation event causes its overexpession (Tsujimoto *et al.*, 1984). *bcl-2*, when overexpressed, prevents the death of cells; in conjunction with the activation of other oncogenes that promote proliferation, such as c-*myc,* this can facilitate tumorigenesis (Vaux *et al.*, 1988). p53, which can cause cell death in response to DNA damage, is one of the most mutated genes in many types of cancers, and people who inherit mutations in p53 are particularly susceptible to developing tumors (Hollstein *et al.*, 1991; Levine *et al.*, 1991; Olivier *et al.*, 2010; Surget *et al.*, 2014). Many cancer therapeutics target the cell death pathway to promote apoptosis of cancer cells, so resistance to apoptosis both contributes to tumorigenesis and resistance to treatment (Goldar *et al.*, 2015; Koff *et al.*, 2015; Plati *et al.*, 2011).

Mutations that prevent cell death might cause autoimmune disease. Mice defective in pro-apoptotic genes Fas or FasL develop Systemic Lupus Erythematosus (SLE)-like disease, likely because autoreactive or improperly-developed T-cells cannot be induced to undergo apoptosis (Cohen and Eisenberg, 1991; Nagata, 2006; Nagata and Suda, 1995). Mutations that inhibit the engulfment of apoptotic corpses are also associated with autoimmune disorders. Patients with the autoimmune disease SLE are often found with unengulfed cell corpses, and their macrophages have reduced phagocytic activity (Gaipl *et al.*, 2007). Mice defective in MFG-E8, a bridging protein that facilitates phagocyte recognition and engulfment of apoptotic cells, develop symptoms similar to SLE (Hanayama *et al.*, 2004), suggesting that defects in engulfment could be sufficient to cause autoimmune disorders. Unengulfed dying cells might undergo a process called secondary necrosis, in which they lyse, causing inflammation and releasing intracellular components (Wyllie *et al.*, 1980). Therefore, proper control of cell death as well as cell corpse clearance are critical to prevent disease.

Regulation of programmed cell death in the nematode *C. elegans* and other organisms

5. *C. elegans* is a useful organism for genetic studies of programmed cell death

Much of the work that has been done to elucidate the genetic control of programmed cell death has used the roundworm *C. elegans*. There are many

features of *C. elegans* that make it a powerful experimental organism, particularly for genetic studies (Brenner, 1974). *C. elegans* is androdioecious, consisting of males and hermaphrodites, which are capable of self-fertilizing or mating with males. The presence of self-fertilizing hermaphrodites streamlines genetic screens and allows for the isolation of homozygous mutants without mating, and the presence of males allows for genetic crosses. The life cycle of *C. elegans* is short; animals hatch from eggs and become fertile adults in about three days. This timing can be manipulated by starvation or changes in temperature, which furthers the organism's experimental power by allowing the researcher to synchronize animals at a given stage or to pause, speed up, or slow down development as experiments demand. But perhaps most advantageous for use in studies of cell death is the fact that *C. elegans* has an essentially invariant pattern of cell division, which has been thoroughly described (Kimble and Hirsh, 1979; Sulston and Horvitz, 1977; Sulston *et al.*, 1980, 1983). Adult hermaphrodites each contain 959 somatic nuclei and during development generate an additional 131 cells that undergo programmed cell death (Sulston and Horvitz, 1977; Sulston *et al.*, 1983). Critically, the identity, location, and timing of these cell deaths are known, so it is possible to screen for mutants, in which cells inappropriately die, survive, or fail to be degraded after dying.

C. elegans cell deaths can be easily observed by Nomarski optics (Sulston and Horvitz, 1977). Living cell nuclei are visible as slight indentations, often speckled (like pepperoni) in the case of neurons or with a large raised nucleolus (like a fried egg) in the case of many other cell types such as hypoderm, intestine, and muscle (Figure 1). During the cell death process, the cytoplasm gradually becomes more

refractile, condensed and round. Eventually, the nucleus becomes refractile as well and the entire cell appears as a flat and refractile disc; this is the characteristic cell corpse appearance (Figure 1). After some time, the refractility lessens and the cell corpse shrinks as it is degraded and eventually disappears.

Genetic screens using Nomarski optics allowed for the identification of cell death defective (Ced) mutants, in which the appearance or number of cell corpses was altered. For example, *ced-1* and *ced-2* mutants each accumulate too many cell corpses (Hedgecock *et al.*, 1983). Electron microscopy showed that these corpses failed to be engulfed (Hedgecock *et al.*, 1983), and further screens for older embryos with an accumulation of undegraded corpses resulted in the identification of several more genes involved in the engulfment of cell corpses (Ellis *et al.,* 1991). Suppressor screens to find mutations that prevented the accumulation of corpses in engulfment mutants allowed the identification of *ced-3*, which is required for the execution of the cell death pathway (Ellis and Horvitz, 1986). Further screens allowed for the identification of *ced-4* and *egl-1*, which are also required generally for cell death; *ced-9,* which antagonizes the cell death pathway; and other genes that modify the cell death process, such as *ced-8* and *ced-11* (Conradt and Horvitz, 1998; Ellis and Horvitz, 1986; Hengartner *et al.*, 1992; Stanfield, 1999; Stanfield and Horvitz, 2000). The isolation and functions of the core cell death genes and the function of their homologs in other organisms will be discussed in further detail below.

6. Execution pathway in *C. elegans*: *ced-3*

ced-3 was isolated in a screen for suppressors of the *ced-1* mutant, which accumulates corpses due to a defect in corpse clearance (Ellis and Horvitz, 1986).

Loss-of-function mutations in *ced-3*, alone or with *ced-1*, result in a loss of almost all cell corpses. Instead, cells that would normally die remain alive and sometimes differentiate to a fate similar to their cellular "sisters" or "aunts." For example, V5.paapp is a cell that normally dies, but in *ced-3* animals the undead V5.paapp cell is sometimes dopaminergic, like its aunt V5.paaa (Ellis and Horvitz, 1986). The undead M4-sister in a *ced-3* animal usually expresses $P_{ceh\text{-}28}$*::gfp*, like its sister, the M4 neuron (Hirose *et al.,* 2010). *ced-3* was shown to act cell-autonomously during cell death; in mosaic animals in which some cells contained functional *ced-3* and others did not, deaths only occurred in cells with functional *ced-3* (Yuan and Horvitz, 1990). Thus, apoptotic deaths appeared to be cell "suicides."

The sequence of *ced-3* was determined, and it was found to be similar to that of the gene encoding Interleukin-1β-Converting Enzyme, a cysteine protease that cleaves proteins after aspartic acid and can cause cell death (Miura *et al.,* 1993; Yuan *et al.,* 1993). Such proteins are now called "caspases." *ced-3* and other caspases are created as zymogens and have prodomains that typically must be removed before the caspases can function as proteases (Degterev *et al.,* 2003; Ramage *et al.,* 1995; Yamin *et al.,* 1996). When activated, CED-3 can cleave a number of targets in the cell leading to the cell's death (Taylor *et al.*, 2007). It is unclear exactly which caspase-targets ultimately lead to the demise of the cell. If cleavage of only a few key substrates was sufficient to cause cell death, mutations that cause altered caspase recognition sites of these substrates might be able to block cell death and would likely have been isolated during cell-death screens. Since no such mutations have been isolated, it is likely that death occurs as the result of many proteins being

cleaved. It is estimated that 3% of the *C. elegans* proteome, about 800 proteins, are targets of CED-3 cleavage, including actin, tubulin, myosin light chain, chaperones, calreticulin, and the anti-apoptotic protein CED-9 (Taylor *et al.*, 2007).

There are a small number of programmed cell deaths in *C. elegans* that do not require *ced-3*. The linker cell death occurs by a non-apoptotic program, which exhibits non-apoptotic morphology and does not require *ced-3* but does require other factors, such as the polyglutamine-repeat gene *pqn-41* (Abraham *et al.,* 2007; Blum *et al.,* 2012; Ellis and Horvitz, 1986). Although apoptosis is dispensible in *C. elegans* as evidenced by the viability of *ced-3* mutants (Ellis and Horvitz, 1986), the death of the linker cell is required for male fertility, as the death opens a passage between the vas deferens and the cloaca (Kimble and Hirsh, 1979). Perhaps the importance of this cell death to the fitness of the male *C. elegans* could have contributed to the alternative control of its death. In addition, a small, variable number of cells die even in caspase-deficient animals, suggesting that cells can stochastically die by other mechanisms (Shaham *et al.,* 1999; Denning *et al.*, 2013). How this might occur is unknown. Another mystery of *ced-3* is that animals containing a deletion allele that spans the protease domain have a weaker cell death defect than animals containing one of many other seemingly "weaker" mutations, such as missense alleles (Shaham *et al.,* 1999). Perhaps the truncated protein produced by the deletion allele might interfere with other factors that contribute to cell death, but this has not been analyzed.

7. Execution pathway in *C. elegans*: *ced-4*

Like *ced-3*, mutations in *ced-4* were found to block essentially all instances of programmed cell death (Ellis and Horvitz, 1986; Yuan and Horvitz, 1992). *ced-4* was isolated as a suppressor of an egg-laying defect caused by the inappropriate death of the hermaphrodite-specific neuron (HSN), which is required for egg laying. By preventing cell death broadly, mutation in *ced-4* also prevented the ectopic death of the HSN, which restored egg-laying function. As *ced-4* is required for the execution of the cell death program, mutations of *ced-4* are able to block death even in a cell that has been specified to die. Initially, *ced-4* and *ced-3* were unable to be ordered in a genetic pathway through epistasis experiments because they had identical loss-of-function mutant phenotypes. However, ectopic expression experiments provided a way to order them: expression of *ced-3* in the absence of *ced-4* was able to kill a cell, but expression of *ced-4* was unable to kill without *ced-3* (Shaham and Horvitz, 1996a). These findings suggested that *ced-4* might act upstream of *ced-3.* More recently, crystal structures have been determined for CED-4 (Qi *et al.*, 2010). CED-4 creates an octomeric ring structure, called an "apoptosome," which contains a hutch-shaped space that binds to a dimer of CED-3. This complex facilitates the autocatalytic activation of CED-3; presumably the zymogen of CED-3 has weak activity and can cleave its partner when held in close proximity (Huang *et al.*, 2013; Yang *et al.*, 1998). Activated CED-3 has more activity in the presence of CED-4, suggesting that the apoptosome and CED-3 might stay together as a complex and function as a holoenzyme (Huang *et al.*, 2013).

Although *ced-4* plays a large role in facilitating apoptosis, it has an anti-apoptotic function as well (Shaham and Horvitz, 1996b). *ced-4* is alternatively spliced to create an abundant short isoform and a less-abundant long isoform. When ectopically expressed, the long isoform can prevent cell death. The SR protein kinase SPK-1 might promote *ced-4L* alternative splicing *in vivo* to prevent cell death, and *spk-1* mutants have extra cell death, presumably because of the lack of antiapoptotic CED-4L (Galvin *et al.*, 2011). How the relative expression of *ced-4S* and *ced-4L* is regulated in living and dying cells and how these two isoforms interact is unknown.

8. Execution pathway in *C. elegans*: *ced-9*

The allele *n1950* was isolated during a screen for mutations that prevent programmed cell death (Hengartner *et al.*, 1992). *n1950* defined a new gene, *ced-9.* Unlike alleles of *ced-3* and *ced-4*, which block cell death recessively, *n1950* blocks cell death dominantly. *n1950* was determined to be a gain-of-function allele, as it is dominant and a deletion spanning the *ced-9* locus does not prevent cell death. To isolate loss-of-function alleles of *ced-9*, a screen for *cis*-dominant suppressors was done. Interestingly, loss-of-function alleles of *ced-9* caused ectopic cell death (during embryogenesis and larval development) and maternally-rescued lethality. The ectopic death of cells in *ced-9(lf)* mutant animals is blocked by *ced-3* or *ced-4* mutation, demonstrating that the ectopic cell deaths in *ced-9(lf)* mutants are apoptotic, and that *ced-9* acts upstream of *ced-3* and *ced-4* to prevent cell death. Thus, it seems that many, if not all, cells are poised to die unless rescued by the activity of *ced-9.*

Further research demonstrated that CED-9 localizes to the mitochondria and binds to CED-4 dimers, sequestering them from interacting with and facilitating the activation of CED-3 (Chen *et al.*, 2000; Yan *et al.*, 2005). A recent study, which used higher-resolution imaging, argues that CED-9 and CED-4 do not actually co-localize and thus CED-9 might not directly regulate CED-4 in the way previously thought (Pourkarimi *et al.*, 2012). However, this study largely examined radiation-induced germ cell deaths instead of somatic cell deaths; mostly analyzed the localization of a CED-4::GFP fusion rather than detecting endogenous, unmodified CED-4 protein; and did not distinguish between CED-4S and CED-4L isoforms, so it is unclear if these results are general and represent the true localization of CED-4S.

Though CED-9 has a strong anti-apoptotic function, it also appears to have a weaker pro-apoptotic activity: weak *ced-3(rf)* mutants have more cell death than *ced-9(lf); ced-3(rf)* double mutants (Hengartner and Horvitz, 1994a). The role of this pro-apoptotic activity during the programmed cell death process remains to be elucidated. Perhaps CED-9 can sequester anti-apoptotic CED-4L to promote cell death.

9. Execution pathway in *C. elegans*: *egl-1*

The most upstream member of the core cell-death pathway in *C. elegans* is the pro-apoptotic gene *egl-1* (Conradt and Horvitz, 1998). Transcriptional control of *egl-1* functions to integrate developmental cues to determine if a cell should live or die (Conradt and Horvitz, 1999). Reflecting this, the *egl-1* gene contains extensive regulatory regions both 5' and 3' to the gene, where transcription factors can

combinatorially bind to control its expression (Hirose *et al.,* 2010; Liu *et al.,* 2006; Nehme *et al.,* 2010; Thellmann, 2003).

This paradigm can be illustrated by the first mutant of *egl-1* to be isolated. Interestingly, this mutant was isolated in a screen for egg-laying defective (Egl) animals (Trent *et al.*, 1983). In wild-type animals, the sex-determination pathway results in high levels of the transcription factor TRA-1 in hermaphrodites and low levels in males (Hodgkin, 1987). TRA-1 binds downstream of the *egl-1* gene in the hermaphrodite-specific neurons (HSNs) and prevents *egl-1* expression (Conradt and Horvitz, 1999). In males, TRA-1 is not highly expressed, so *egl-1* is expressed in the HSNs, and they undergo programmed cell death. In the first mutant allele of *egl-1* to be isolated, the TRA-1 binding site is mutated, which prevents TRA-1 from repressing *egl-1* expression in the HSNs even in the hermaphrodite. The HSNs inappropriately die, and the animal becomes Egl.

More mutants that alter the transcriptional regulation of *egl-1* have been isolated. Rather than globally affecting cell death, as mutations in the coding-region do, mutations in the regulatory regions of *egl-1,* or in the genes encoding transcription factors that control *egl-1* expression, typically alter the deaths of only a small number of cells (Nehme and Conradt, 2009). For example, gain-of-function mutations in *ces-1* and loss-of-function mutations in *ces-2* prevent the death of the NSM and I2 sisters, but do not affect the deaths of other cells in the head or ventral cord (Ellis and Horvitz, 1991). A mutation approximately 5 kb upstream of *egl-1* prevents the death of the M4-sister by preventing the binding of EYA-1 and CEH-34, but this mutation does not affect cell death globally (Hirose *et al.*, 2010).

There is likely post-transcriptional regulation of *egl-1* as well. There are predicted microRNA binding sites in the *egl-1* 3' UTR, which can mediate deadenylation in a reporter assay (Wu *et al.*, 2010). Furthermore, at least two transcriptional reporters for *egl-1* are expressed in a pattern that is not identical to the pattern of cell deaths (unpublished observation). These reporter constructs, which do not contain the *egl-1* 3' UTR, are expressed in many cells that do not die and are not expressed in all dying cells. This mismatch between *egl-1* reporter expression and the pattern of cell death might be a result of the transcriptional reporters not containing the entire regulatory domains of *egl-1* or the 3' UTR, preventing miRNA regulation. It might also indicate that EGL-1 must accumulate to a certain threshold before it causes the death of the cell, and that this threshold varies between cells. Thus, cells with a high threshold might survive despite accumulating small amounts of EGL-1, while cells with a low threshold might die before accumulating detectable amounts of EGL-1. Such a threshold might be determined by the expression level of other cell death genes.

Once expressed in a cell, the EGL-1 protein binds to CED-9 in a way that disrupts its interaction with CED-4 (del Peso *et al.*, 1998). This disruption releases CED-4 to facilitate the activation of CED-3, resulting in the death of the cell. The gain-of-function allele *ced-9(n1950),* which causes weaker binding between CED-9 and EGL-1 and prevents the release of CED-4 when *egl-1* is expressed, blocks most somatic cell deaths, underscoring the importance of *egl-1* in controlling cell death in *C. elegans* (del Peso *et al.*, 2000).

10. Execution pathways in other organisms

Unlike *C. elegans*, which has an essentially invariant pattern of cell divisions and no somatic cell proliferation in adulthood, vertebrates have continued cell proliferation, which is balanced by a more elaborate cell death pathway (Degterev and Yuan, 2008). Mammals have both an intrinsic suicide pathway and an extrinsic induced suicide pathway, which allows signals from other cells to induce a cell-autonomous suicide program.

The structure of the intrinsic cell death pathway in mammals is similar to the *C. elegans* suicide pathway and contains homologs to *egl-1, ced-9, ced-4,* and *ced-3* (Figure 2)(Conradt, 2009). EGL-1 and CED-9 are Bcl-2 family proteins with strong pro- and anti-apoptotic activity, respectively (Conradt and Horvitz, 1998; Hengartner and Horvitz, 1994b); mammals have a number of pro- and anti-apoptotic Bcl-2 family members. Bcl-2 proteins Bid, Bim, Puma, Bmf, Hrk, Bik, Noxa, and Bad are pro-apoptotic BH3-only proteins like EGL-1 (Boyd *et al.*, 1995; Inohara *et al.*, 1997; Luo *et al.*, 1998; Nakano and Vousden, 2001; Oda *et al.*, 2000; O'Connor *et al.*, 1998; Puthalakath *et al.*, 2001; Yang *et al.*, 1995). BH3-only proteins contain only the third Bcl-2 homology domain, which is required for their ability to induce cell death (Chittenden *et al.*, 1995). Bcl-x_L, Bcl-2, Bcl-w, A1, Mcl-1, and Bcl-B are anti-apoptotic Bcl-2 family members (Boise *et al.*, 1993; Gibson *et al.*, 1996; Hockenbery *et al.*, 1993; Ke *et al.*, 2001; Kozopas *et al.*, 1993; Lin *et al.*, 1993). These two groups of Bcl-2 family proteins antagonize each other through direct interactions, and the balance of these classes will determine if Bax and Bak become activated (Westphal *et al.*, 2014; Willis and Adams, 2005). Bid, Bim, Puma, Noxa, and possibly other BH3-

only proteins can directly activate the pro-apoptotic Bcl-2 family members Bax and/or Bak (Willis and Adams, 2005; Zheng *et al.*, 2015). When activated, Bax and Bak can form oligomers at the mitochondrial outer membrane and create pores, causing mitochondrial outer membrane permeability (MOMP) and release of proapoptotic factors from the mitochondria such as cytochrome c (Czabotar *et al.*, 2014; Tait and Green, 2010). Cytochrome c binds to Apaf-1, the homolog of CED-4, and pro-caspase-9 to form an apoptosome with 7-fold symmetry that facilitates the activation of caspase-9 (Jiang and Wang, 2000; Yuan and Akey, 2013). This mechanism is similar to the way that the apoptosome of CED-4 and CED-3 facilitates the activation of CED-3 (as described above).

Caspase-9 is an initiator caspase; initiator caspases have long N-terminal domains to facilitate their interactions with the apoptosome (caspases-2, -8, and -10 are also mammalian initiator caspases) (Riedl and Shi, 2004). Once activated, initiator caspases can cleave and activate the effector caspases (in mammals, caspases-3, -7, and -6), which are able to cleave substrates in the cell to cause cell death. The activity of caspases in mammals is subject to an additional level of control that is not present in *C. elegans*; inhibitor of apoptosis (IAP) proteins can inhibit caspases-3, -7, and -9 (Salvesen and Duckett, 2002). IAPs act as competitive inhibitors of effector caspases-3 and -7 and can bind to monomers of caspase-9 to prevent them from forming functional dimers (Maier *et al.*, 2002; Shiozaki *et al.*, 2003). The inhibition of apoptosis by IAPs can be relieved by the activity of proapoptotic proteins that are released from the mitochondria during MOMP, such

as SMAC/DIABLO, which can bind to IAPs and disrupt their interactions with caspases (Chai *et al.*, 2000; Du *et al.*, 2000; Verhagen *et al.*, 2000).

In the extrinsic pathway, extracellular ligands bind to death receptors in a way that recruits a death-inducing signaling complex (DISC) that can promote caspase activation (Guicciardi and Gores, 2009; Jin and El-Deiry, 2005). Fas ligand binds Fas (also called Apo-1 or CD95) to cause conformational changes that facilitate the recruitment of FADD and promote the formation of a DISC that promotes caspase-8 cleavage and subsequent activation of caspases-3 and -7 (Wajant, 2002). Caspase-8 can cleave and activate Bid to promote the intrinsic apoptotic pathway as well (Li *et al.*, 1998). TNF binding to its receptor TNF-R1 allows for the assembly of TRAF2, RIP1 kinase, and IKK (Sedger and McDermott, 2014). This complex activates downstream signaling processes such as JNK and NF-κB, which can actually mediate cell survival. However, it is also possible for the TNF/TNF-R1 complex to become internalized and associate with FADD and caspase-8, resulting in the activation of caspase-8 and the death of the cell. TRAIL and its receptors can induce cell death in a way similar to FasL and Fas, or can activate JNK and NF-κB in a way similar to TNF and its receptor (Pitti *et al.*, 1996; Wang and El-Deiry, 2003; Wiley *et al.*, 1995). FasL, TNF, or TRAIL signaling can also induce a caspase-independent form of cell death that is necrotic in morphology, which is mediated through RIP kinases RIP1 and RIP3 (Holler *et al.,* 2000; Zhang *et al.,* 2009). This form of cell death is regulated as is apoptosis, but it has necrotic morphology and thus is called necroptosis (Degterev *et al.,* 2005).

The cell death pathway in *Drosophila* is of intermediate complexity compared to those of *C. elegans* and mammals (Figure 2) (Denton *et al.,* 2013). Unlike in *C. elegans* and mammals, where proapoptotic signals are transduced through Bcl-2 family member proteins, death in the fly is initiated through activation of *rpr, hid,* and *grim,* which encode IAP antagonists (Goyal *et al.,* 2000; Ryoo *et al.,* 2002; Wang *et al.,* 1999; Yoo *et al.,* 2002; Zachariou *et al.,* 2003). These three genes happen to be genetically linked, so a single deletion was able to eliminate function of all three and prevent almost all developmental cell deaths (White *et al.,* 1994). Further parsing of the deleted region revealed each of the three genes (Chen *et al.,* 1996; Grether *et al.,* 1995; White *et al.,* 1994). *rpr* and *grim* are expressed only in dying cells, and expression and activity of HID is regulated at many levels (Bilak and Su, 2009; Chen *et al.,* 1996; Grether *et al.,* 1995; White *et al.,* 1994).

Of the four IAPs in *Drosophila,* Diap1 plays the biggest role in preventing apoptosis by preventing the activation of the initiator caspase Dronc and targeting it for degradation (Hay *et al.,* 1995; Kaiser *et al.,* 1998; Meier *et al.,* 2000; Wilson *et al.,* 2002). The initiator caspase Dronc is activated by an apoptosome containing Dark/Ark, which is homologous to Apaf-1 and CED-4 (Daish *et al.,* 2004; Kanuka *et al.,* 1999; Rodriguez *et al.,* 1999; Yuan *et al.,* 2011; Zhou *et al.,* 1999). As in *C. elegans*, the fly apoptosome does not require cytochrome c for assembly and caspase activation (Yu *et al.*, 2006a). Drice is the main effector caspase in fly apoptosis and corpse clearance, with Dcp-1 playing a redundant role in some cells (Akagawa *et al.,* 2015; Fraser and Evan, 1997; Song *et al.,* 2004; Xu *et al.,* 2006). While *Drosophila* contains two predicted Bcl-2 family member genes, they appear dispensable for

developmental cell death (Sevrioukov *et al.*, 2007). Thus, cells in the fly might be poised for death with active caspase held in check by Diap1 until developmental cues cause RPR, HID, and GRIM to free Dronc from Diap1's inhibition.

Corpse clearance in *C. elegans* and other organisms

The cell corpses generated by programmed cell death are engulfed by neighboring cells or by professional phagocytes (depending on the organism and tissue) and degraded (Poon *et al.*, 2014; Savill and Fadok, 2000). This process happens rapidly, such that only a small number of cell corpses are visible even in tissues where many deaths are occurring. It has been speculated that this lack of accumulation of cell corpses caused the phenomenon of programmed cell death to be historically overlooked and its prevalence to be underestimated (Jacobson *et al.,* 1997). In *C. elegans*, there is evidence that engulfment can occur in parallel to the cell death execution process; a cell can begin to be engulfed even before the cell division that generates it has completed (Robertson and Thomson, 1982).

In theory, there are multiple steps that are required for a corpse to be removed. A dying cell must undergo changes that allow it to be recognized as a dying cell requiring disposal. These signals must be recognized by an engulfing cell, and these signals must be transduced in a way that results in the rearrangement of the engulfing cell. The engulfing cell extends pseudopods to surround and engulf the cell corpse. Genes have been identified that act in each of these steps, which will be discussed below.

11. "Eat me" signals

Phosphatidylserine (PS) is the most widely-accepted "eat me" signal in *C. elegans* and other organisms (Fadok *et al.*, 1998a). PS is retained on the inner leaflet of the cell membrane in living cells and externalized on the surface of apoptotic cells, at least in part, by the activity of Xkr8 protein in mammals and its homolog *ced-8* in *C. elegans* (Chen *et al.*, 2013; Suzuki *et al.*, 2013) and possibly *ced-7* in *C. elegans* (Mapes *et al.*, 2012). In some studies of *C. elegans* and mammals, PS exposure appears to be sufficient for cell engulfment (Darland-Ransom *et al.*, 2008; Segawa *et al.*, 2014). However, there are likely other "don't eat me" signals that can prevent the engulfment of living cells in many cases, such as CD47, which is necessary for the protection of erythrocytes (Oldenborg *et al.*, 2000). Other eat-me signals in mammals include calreticulin, thrombospondin, intercellular adhesion molecule-1 or -3, oxidized low-density lipoprotein or complement C1q, which are recognized by a variety of receptors on the engulfing cell (Hawkins and Devitt, 2013; Hochreiter-Hufford and Ravichandran, 2013; Ravichandran and Lorenz, 2007).

12. Recognition, signal transduction, and engulfment in *C. elegans*

In *C. elegans*, there are two major parallel pathways (*ced-2/5/10/12* and *ced-1/6/7*) that might converge at *ced-10* and a third parallel pathway that contributes to a lesser extent (Figure 3) (Ellis *et al.,* 1991; Hurwitz *et al.,* 2009; Kinchen *et al.,* 2005). Even in strong mutants or double-mutants for engulfment, a small fraction of cells are still engulfed and rapidly degraded; a mutant that is completely defective in engulfment has never been isolated (Hedgecock *et al.*, 1983; Kinchen *et al.*, 2005).

One reason for the lack of an engulfment-null mutant could be that a mutation that completely eliminates engulfment activity might also cause lethality. However, screens have been designed to isolate essential genes with a role in engulfment (Yu *et al.*, 2006b), and multiple essential genes with a role in engulfment have been identified. More likely, the lack of an engulfment-null mutant is due to high levels of redundancy in the engulfment pathways. The genetic control of engulfment continues to be an active area of research, with new engulfment genes being identified often. The exact mechanisms of what makes a cell competent to engulf and how it recognizes its apoptotic neighbor are still open questions.

The *ced-2/5/10/12* pathway

One branch of the engulfment pathway in *C. elegans* is historically defined by the genes *ced-2, ced-5, ced-10* and *ced-12* (Figure 3). Multiple lines of evidence suggest that these genes act in the same genetic pathway. When double mutants are created between any two of these genes, they do not accumulate more corpses than a complete loss-of-function of one of these genes, but when double mutants between these genes and genes of the other parallel pathway are created, they do have an enhanced number of persistent corpses (Ellis *et al.,* 1991). Furthermore, mutations of each of these genes causes a defect in the migration of the distal tip cells in addition to their defect of cell corpse clearance (see below); mutations in the genes of the other engulfment pathway do not (Wu and Horvitz, 1998a; Gumienny *et al.*, 2001; Reddien and Horvitz, 2000). Thus, these four genes seem to be acting in the same process.

These four genes have been cloned, and the domains contained in the proteins they encode suggest a molecular mechanism for control of engulfment. *ced-2* encodes a protein with a Src-Homology-2 (SH2) domain and two Src-Homology-3 (SH3) domains (Reddien and Horvitz, 2000). SH2 domains typically bind to phosphorylated tyrosine residues, so it is likely that CED-2 is an adaptor protein that transduces an engulfment signal by binding to a yet-unidentified signaling protein at the cell membrane that is phosphorylated when a neighboring apoptotic cell is recognized (Reddien and Horvitz, 2000). SH3 domains bind to proline-regions, one of which can be found in CED-5 (Wu and Horvitz, 1998a). CED-5's own SH3 domain might interact with a proline-rich region of CED-12 (Zhou *et al.*, 2001a). CED-2, CED-5, and CED-12 and their mammalian counterparts interact *in vitro,* which can be seen through co-immunoprecipitation experiments and yeast two- or three-hybrid studies (Gumienny *et al.*, 2001; Reddien and Horvitz, 2000; Wu *et al.*, 2001; Zhou *et al.*, 2001a). These interactions bring CED-5 to the cell membrane (Gumienny *et al.*, 2001). CED-12 itself is likely localized to the cell membrane through its Pleckstrin Homology (PH) domain, which binds to phosphatidylinositol lipids in the membrane (Zhou *et al.*, 2001a). CED-5 and CED-12 likely act as a bipartite guanine exchange factor (GEF) for the Rac GTPase CED-10, facilitating CED-10's binding to GTP, which allows it to be active and promote the restructuring of the cytoskeleton (Brugnera *et al.*, 2002; Gumienny *et al.*, 2001).

The *C. elegans* genome encodes two other Rac-like proteins, MIG-2 and RAC-2/3, which play minor roles in engulfment that can only be detected in a sensitized background (Lundquist *et al.*, 2001). The mammalian homologs of *unc-73* and *mig-2*

were found to interact with the homologs of *ced-5, ced-10,* and *ced-12; unc-73* and *mig-2* were subsequently studied and found to act upstream of *ced-5* and *ced-12* in parallel to *ced-2* (deBakker, Colin *et al.*, 2004). MIG-2 is a Rho-family GTPase, and UNC-73 is a RhoGEF that activates MIG-2. The active MIG-2 might promote CED-12 docking to the plasma membrane. The activity of *unc-73* and *mig-2* is minor; these mutants cause visible engulfment defects only in a sensitized background, such as in animals containing a weak mutation in *ced-2, ced-5*, *ced-10* or *ced-12.* Thus, while *unc-73* and *mig-2* appear to act upstream to activate CED-5 and CED-12 in parallel to *ced-2*, they are likely not the major activating factors (deBakker, Colin *et al.*, 2004).

A role for *mtm-1* in engulfment was independently identified by two labs, in an RNAi screen for suppression of *ced-1* defects (Zou *et al.*, 2009) and in a genetic screen for mutants with enhanced engulfment that suppressed *ced-6* mutation (Neukomm *et al.*, 2011a). *mtm-1* belongs to a family of polyphosphoinositide 3-phosphatases and has phosphatase activity *in vitro* towards phosphoinositide 3-phosphate (PtdIns3*P*) and PtdIns(3,5)P_2 (Neukomm *et al.*, 2011a). *mtm-1* functions upstream of the *ced-5/10/12* pathway (Neukomm *et al.*, 2011a; Zou *et al.*, 2009). Zou *et al.* found that *mtm-1* acts in parallel to *ced-2* (2009), but Neukomm *et al.* found that *mtm-1* acts upstream of *ced-2* (2011a). This discrepancy might be explained by the fact that they examined different cells or that Zou *et al.* used RNAi while Neukomm *et al.* used genetic loss-of-function alleles. The PH domain of CED-12 can bind polyphosphoinositides, including PtdIns(3,5)P_2, so it is likely that *mtm-1* represses engulfment by removing the phosphoinositide-dependent recruitment or activation of CED-12 and CED-5 (Neukomm *et al.*, 2011a). Indeed, *mtm-1* RNAi does

not suppress the number of cell corpses in *ced-1, ced-6,* or *ced-7* mutants when the phosphatidylinositol 3-kinases *vps-34* and *piki-1* are also mutated (Zou *et al.*, 2009). Interestingly, RNAi of *mtm-1* also results in the delayed degradation of engulfed germ cell corpses (Neukomm *et al.*, 2011a). Thus, *mtm-1* might be required at two steps in the clearance process, for both engulfment and degradation.

srgp-1 was identified in an RNAi screen of all *C. elegans* genes containing a predicted RhoGAP domain looking for suppression of engulfment defects associated with engulfment mutants (Neukomm *et al.*, 2011b). SRGP-1 binds specifically to the active form of CED-10 and promotes GTP-hydrolysis of the human homolog of CED-10 *in vitro*. Mutants of *srgp-1* have hyperactive engulfment, presumably because CED-10 stays active for longer.

Another negative regulator of CED-10 is PDR-1. The homolog of Parkin, *pdr-1* encodes an E3 ubiquitin ligase. Its potential role in engulfment was studied in *C. elegans* after it was found that Parkin and Rac1 interact in aged human brains (Cabello *et al.*, 2014). *pdr-1* was found to negatively regulate engulfment, resulting in reduced kinetics of engulfment and an accumulation of corpses. *pdr-1* mutation also suppresses other phenotypes of *ced-10* mutation, such as the distal tip cell migration defect. PDR-1 interacts with and ubiquitylates CED-10 *in vitro*, and CED-10 levels are higher in *pdr-1* mutants, suggesting that PDR-1 likely reduces levels of CED-10 *in vivo*.

swan-1 is another negative regulator of *ced-10* and probably *mig-2* (Yang *et al.*, 2006). *swan-1* was identified by a yeast two-hybrid screen; SWAN-1 interacted with the LIM domains of UNC-115, a downstream effector of cytoskeleton rearrangement.

swan-1 mutation suppresses the distal tip cell migration and persistent cell corpse phenotype of a weak allele of *ced-10*, but not a putative null allele, suggesting that it acts upstream of *ced-10* to negatively regulate its function. However, the mechanism of this activity is not known.

ced-10 and its regulators have roles in other cytoskeleton movement-driven processes as well, such as axon and cell migration (Wu and Horvitz, 1998a; Reddien and Horvitz, 2000; Lundquist *et al.*, 2001; Quinn *et al.*, 2008). A striking example of this is the dependence of distal tip cell migration on the genes of the *ced-2/5/10/12* pathway. The distal tip cells (DTCs) are a pair of cells in the hermaphrodite somatic gonad, which migrate in opposite directions in a U-shape away from the vulva along the ventral side and back towards the midbody on the dorsal side of the animal (Kimble and Hirsh, 1979). As the DTCs migrate, the germline proliferates behind them to create the hermaphrodite's bilobed gonad structure. Because the length and shape of the germline is defined by the movements of the DTCs, the germline shape is a trace of the migratory path of the DTCs. In animals with a defect in the *ced-2/5/10/12* pathway, the DTCs sometimes fail to migrate fully and sometimes migrate in inappropriate directions, for example continuing to migrate towards the head or tail instead of turning back to the center of the animal or changing direction multiple times (Wu and Horvitz, 1998a; Reddien and Horvitz, 2000; Lundquist *et al.*, 2001; Gumienny *et al.*, 2001). *ced-10* is expressed in the DTCs and might be acting cell-autonomously to promote their proper migration by promoting cytoskeletal rearrangement within the distal tip cells (Lundquist *et al.*, 2001).

How are signals on a dying cell recognized to activate the *ced-2/5/10/12* pathway? No mutations of signaling molecules or receptors have been identified that cause as strong of a corpse-accumulation phenotype as do mutations in *ced-2, -5, -10,* or *-12.* Instead, there are multiple, minor pathways proposed to facilitate cell corpse recognition and signaling upstream of the *ced-2/5/10/12* pathway, which are described below. This redundancy could explain why mutations in any one of these pathways only weakly inhibits engulfment.

Mutants defective in the Frizzled gene *mom-5*, in addition to displaying defects associated with Wnt signaling such as problems in spindle polarity, have persistent corpses and defects in distal tip cell migration (Cabello *et al.*, 2010). These abnormalities are suppressed by overexpression of *ced-5* or *ced-10*. Mutants of *gsk-3*, which acts downstream of *mom-5* in other Wnt-controlled processes, also contain persistent corpses. In other organisms, GSK-3 interacts with APC. The APC homolog APR-1 was found to act with CED-2 in a yeast two-hybrid screen, and *apr-1* mutants also have persistent corpses. Thus, MOM-5 might act as a receptor for cell corpses, which transduces a signal via GSK-3 and APR-1 to activate CED-2 and the engulfment pathway. It is unclear how MOM-5 might recognize cell corpses.

Although it was once reported that integrins did not play a detectable role in engulfment (Gumienny *et al.*, 2001), it was found that the integrin α subunit *pat-2*, together with RhoGTPase family member *cdc-42* and its activator *uig-1*, is required specifically for engulfment by muscle cells (Hsieh *et al.*, 2012). This specificity might explain why the role of the integrins in engulfment was overlooked before. In hypodermal cells, *pat-2* is dispensable for engulfment, but a different integrin α

subunit, *ina-1* is required (Hsieh *et al.*, 2012). The β integrin *pat-3* functions with both *pat-2* and *ina-1.* Interestingly, the extracellular domains of INA-1 and PAT-2, when expressed ubiquitously by a heat-shock promoter, can recognize some of the same corpses. Yet, expression of INA-1 in muscle cells or PAT-2 in hypodermal cells can only partially rescue a *pat-2* or *ina-1* mutant, respectively. These findings suggest that *pat-2* and *ina-1* function in distinct ways rather than simply being expressed in different tissues or recognizing distinct sets of apoptotic corpses. The extracellular domain of PAT-2 is sufficient for the recognition and binding of apoptotic cells, but the intracellular domain is required to cause CDC-42 clustering around apoptotic cells, presumably by signaling through UIG-1 and its partner UNC-112 (Hsieh *et al.*, 2012; Neukomm *et al.*, 2014). Although Hsieh and colleagues found *pat-2* to act independently of any of the three main engulfment pathways (2012), Neukomm and colleagues found that *cdc-42* appears to act downstream of the *ced-1/6/7* pathway, possibly activating CED-10 indirectly by activating the Rac GEF TIAM-1 (2014). Other studies suggest that *pat-3* and *ina-1* function upstream of the *ced-2/5/10/12* pathway (Hsu and Wu, 2010), with the tyrosine kinase *src-1* linking *ina-1* with *ced-2*. In these studies, *ina-1* requires the phospholipid scramblase *scrm-1* to bind apoptotic cells, so *ina-1* might recognize phosphatidylserine or another yet unidentified moiety on apoptotic cells that is exposed by scramblase function.

A putative phosphatidylserine (PS) receptor was identified in *C. elegans* by homology to one identified first in mammals (Wang *et al.,* 2003). The mammalian putative PS receptor was identified by Fadok and colleagues in 2000. They generated an antibody (mAb217) that bound strongly to human macrophages

induced to recognize PS. The antibody's binding to the activated macrophages was outcompeted by preincubating the macrophages with PS liposomes, suggesting that the antibody might be specific to a receptor for PS. This antibody was then used to screen a phage display library, which identified a conserved protein that, when expressed in a non-phagocytic cell, conferred the ability to bind and engulf apoptotic cells (Fadok *et al.*, 2000). Based on this, Wang *et al.* analyzed the *C. elegans* homolog, *psr-1*, for a role in engulfment (2003). They found a mild delay of engulfment in *psr-1* mutants, and based on double-mutant and overexpression analyses placed it in the *ced-2/5/10/12* pathway. They also found that PSR-1 could interact with CED-5 and CED-12 *in vitro*.

However, the validity of the identification of *psr-1* and its homologs as true phosphatidylserine receptors has been debated (Williamson and Schlegel, 2004; Wolf *et al.*, 2007). Vertebrate experiments do not consistently find defects in engulfment in mutants for PSR, although PSR mutants have gross developmental abnormalities (Böse *et al.*, 2004; Hong *et al.*, 2004; Kunisaki *et al.*, 2015; Li *et al.*, 2003). The PSR gene also contains several nuclear localization signals, and tagged versions of PSR localize to the nucleus, suggesting that PSR might not naturally be a membrane-associated protein (Cui *et al.*, 2004). Importantly, it was found that PSR deletion did not affect binding of the mAb217 antibody, which was originally used to identify PSR, to macrophages (Böse *et al.*, 2004). Thus, the binding of mAb217 during the phage display library screening might have been nonspecific, and the gene that was isolated might not actually encode a phosphatidylserine receptor. More recently, researchers from the lab that originally characterized *psr-1* in *C.*

elegans have reiterated its role in engulfment (Yang *et al.*, 2015). They demonstrate *in vitro* binding to PS and find a strong cell corpse clearance defect of irradiation-induced germ cell corpses. While endogenous PSR-1 was not detectable by antibodies in this study, transgenic PSR-1 appeared to cluster around a small number of germline corpses. Thus, while it seems clear that PSR-1 has some ability to bind PS or other membrane lipids *in vitro* and might be membrane-bound in transgenic experiments, it is unclear where endogenous PSR-1 is naturally localized or how it might promote the engulfment of irradiation-induced germ cell deaths.

The *ced-1/6/7* pathway

The other major branch of the engulfment pathway is defined by the genes *ced-1, ced-6,* and *ced-7* (Figure 3). Among the engulfment genes, *ced-7* is unique in that it is required in both the engulfing and dying cells; the rest only seem to be required in the engulfing cell (Liu and Hengartner, 1998; Wu and Horvitz, 1998b; Zhou *et al.*, 2001b). *ced*-7 encodes an ATP-Binding Cassette transporter, which might affect lipid asymmetry in the cell membrane (Wu and Horvitz, 1998b). Indeed, *ced-7* seems to be required for phosphatidylserine exposure of somatic corpses (Mapes *et al.*, 2012). *ced-7* is required for CED-1 clustering around somatic cell corpses, suggesting that it might act upstream of *ced-1*, perhaps in the cell corpse to display whatever cell corpse markers are recognized by CED-1 (Zhou *et al.*, 2001b). However, in the germline, *ced-7* is not required for CED-1 or CED-6 to cluster around corpses, and phosphatidylserine exposure seems to be promoted by *plsc-1* instead (Venegas and Zhou, 2007).

ced-1 encodes a receptor protein that recognizes cell corpses, although the moiety that it recognizes is unknown (Zhou *et al.*, 2001b). The extracellular domain of CED-1 is sufficient for its clustering around corpses, but the intracellular domain is required for cell clearance (Zhou *et al.*, 2001b). *ced-6* encodes an adaptor protein that can bind to the intracellular domain of CED-1 and might transduce the signal that a corpse has been recognized (Liu and Hengartner, 1998; Su *et al.*, 2002; Zhou *et al.*, 2001b). CED-6 contains a phosphotyrosine-binding domain, and this is sufficient for interaction with CED-1 *in vitro* (Liu and Hengartner, 1998; Su *et al.*, 2002). The intracellular region of CED-1 contains two tyrosine residues that could be phosphorylated and recognized by CED-6 (Zhou *et al.*, 2001b). However, the role of CED-1 phosphotyrosines in engulfment is unclear: while these regions are required for engulfment, their phosphorylation does not seem to be required for interaction with CED-6 (Su *et al.*, 2002; Zhou *et al.*, 2001b). Perhaps CED-6 can bind inactive CED-1, but only transduces a signal when CED-1 is phosphorylated.

In a screen designed to isolate mutant alleles of essential genes with a role in engulfment, several alleles of *dyn-1* were found (Yu *et al.*, 2006b). *dyn-1* alleles that affect the GTPase domain of DYN-1 recessively cause embryonic arrest at the late 4-fold stage and the accumulation of apoptotic corpses. *dyn-1* loss-of-function enhances the loss-of-function phenotype of genes in the *ced-2/5/10/12* pathway but not the *ced-1/6/7* pathway, which suggests that *dyn-1* functions in the latter pathway. Additionally, DYN-1 localizes normally to the pseudopods that surround a corpse in mutants of the *ced-2/5/10/12* pathway but not the *ced-1/6/7* pathway, which suggests that *dyn-1* likely acts downstream of the *ced-1/6/7* pathway. *dyn-1*

and *ced-1*, but not *ced-10* and *ced-12,* are required for the delivery of vesicles to the phagosome pseudopods arms. The delivered vesicles can fuse to the pseudopods and provide a source of membrane to extend the pseudopods. Additionally *dyn-1* mutants display defects in the degradation of corpses that are already engulfed, suggesting that it might also play a role in other processes such as vesicle trafficking or phagosome maturation.

A similar screen facilitated the isolation of mutants of *epn-1* (Shen *et al.*, 2013). *epn-1* mutants accumulate persistent corpses and arrest during late embryogenesis or the first larval stage. EPN-1 is a member of the epsin family, members of which can function as adaptors for clathrin, and it was found that the clathrin heavy chain gene *chc-1* was also required for cell corpse clearance. *epn-1* and *chc-1* mutants have slower engulfment of corpses, and these genes appear to act in the *ced-1/6/7* pathway. Indeed, EPN-1 clustering to pseudopods is dependent on *ced-1* and *ced-6,* and CHC-1 clustering to pseudopods was dependent on *epn-1* as well as the upstream genes *ced-1, ced-6,* and *dyn-1*. In mutants of *epn-1*, *chc-1, ced-6*, or *dyn-1* F-actin was observed to extend more slowly and often retract. Thus, it appears that a *ced-1/6/7/dyn-1/epn-1/chc-1* pathway regulates the assembly of F-actin in phagocytic pseudopods.

In another screen *ttr-52* mutation was found to cause defects in the rate of cell corpse clearance (Wang *et al.*, 2010). TTR-52 contains a secretion signal; it appears to be synthesized in the intestine and secreted, after which it clusters around apoptotic cells. This finding, combined with the observation that it acts in the *ced-1/6/7* pathway, suggested that it might facilitate CED-1's recognition of and

clustering around cell corpses, but this mechanism and what it might bind are open questions.

Additional players in *C. elegans* engulfment

abl-1 and *abi-1* define a third parallel engulfment pathway (Figure 3a) (Hurwitz *et al.*, 2009). Mutation of the Abl homolog *abl-1* partially suppresses the persistent corpse phenotype of most single engulfment mutants in both the *ced-1/6/7* pathway as well as the *ced-2/5/10/12* pathway. *abl-1* does not reduce the number of corpses in mutants defective in both pathways, perhaps because its suppression is too weak to make an impact on such a strong engulfment mutant. In contrast, RNAi knockdown or mutation of *abi-1*, the homolog of the Abl interactor (Abi) proteins, enhanced the persistent corpse phenotype of all engulfment mutants tested, including mutants containing strong loss of function mutations in both engulfment pathways. *abl-1* is not required for *abi-1* function, suggesting that *abl-1* likely acts upstream of *abi-1*. ABI-1 and ABL-1 interact *in vitro*, suggesting that ABL-1 might directly interact with and inhibit ABI-1 to suppress engulfment.

sli-1 was identified as a candidate engulfment gene based on mammalian evidence that its homolog Cbl interacts with the homologs of CED-10 and ABL-1 and can be regulated by the homolog ABI-1 (Anderson *et al.*, 2012). Like *abl-1,* by itself, *sli-1* mutation has no effect on engulfment, however it partially suppresses the cell corpse phenotype of engulfment mutants, especially mutants of the *ced-1/6/7* pathway. *sli-1* fails to suppress the cell corpse phenotype of *ced-2/5/12* mutations, but does suppress the distal tip cell migration defect of these mutants. *sli-1* also seems to act in a different pathway than *abl-1* and *abi-1*. Thus, *sli-1* might function in

a fourth parallel pathway, or it might act in parallel to *ced-2/5/10/12* for distal tip cell migration but in the *ced-2/5/10/12* pathway for engulfment.

The role of *pgrn-1* in cell death was studied because its mammalian homolog, progranulin, is associated with frontotemporal lobar degeneration (Kao *et al.*, 2011). It was found that *pgrn-1* mutant embryos contained fewer corpses and corpses were cleared faster. *prgn-1* mutation does not suppress the cell corpse accumulation in larval heads of any engulfment mutant tested, which was interpreted to mean that *prgn-1* required intact engulfment to exert its activity. However, this is also consistent with the interpretation that *prgn-1* affects the rate of engulfment and/or degradation without affecting the initiation of engulfment. Even in engulfment mutants some corpses are engulfed and degraded, and the kinetics of this seem roughly normal, suggesting that the ability to initiate, not complete, engulfment is a limiting factor in engulfment mutants (Hedgecock *et al.*, 1983). Thus, if *prgn-1* affects only the kinetics of engulfment or degradation, it would not be expected to have a substantial effect on the persistence of corpses after hatching, a fairly late time point compared to when embryonic deaths occur. *prgn-1* mutant macrophages had increased phagocytic activity *in vitro*; these data are consistent with an increased speed of engulfment, but not degradation, in *prgn-1* mutants (Kao *et al.*, 2011). Thus, it appears that *prgn-1* normally functions to decrease the kinetics of engulfment (but perhaps does not affect initiation of engulfment). The mechanism of *prgn-1* function in this process has not been investigated.

Uniting the pathways

Originally, the *ced-1/6/7* pathway was thought to act in parallel to the *ced-2/5/10/12* pathway, however newer evidence suggests that *ced-10* might be downstream of both pathways (Kinchen *et al.*, 2005). *ced-10* is an essential gene; null mutants suffer from embryonic lethality, so partial loss-of-function mutants have been used in epistasis experiments (Ellis *et al.,* 1991; Lundquist *et al.,* 2001). This complicates the interpretation of pathway analysis experiments, since partial loss-of-function mutants can potentially enhance mutations in genes both in the same or in parallel pathways. The idea that *ced-10* might act downstream of both engulfment pathways is supported by multiple lines of evidence: overexpression of *ced-10* suppresses mutations in either of the two pathways, mutations in genes in either pathway disrupt the formation of actin halos around cell corpses, and double-mutant analysis using a maternally-rescued null mutation of *ced-10* found that mutations in either pathway did not enhance the engulfment phenotype of *ced-10* null animals (Kinchen *et al.*, 2005). Interestingly, a few corpses are still engulfed and degraded even in these *ced-10* null animals, emphasizing that while these two pathways are majorly responsible for engulfment of cell corpses, there are other processes involved as well (Kinchen *et al.*, 2005).

However, other genes, such as *dyn-1, epn-1,* and *chc-1* seem to act downstream of *ced-1/6/7* but not *ced-10* (Shen *et al.*, 2013; Yu *et al.*, 2006b). These genes seem to regulate actin dynamics (Shen *et al.*, 2013), so the finding that either pathway can disrupt the formation of actin halos around corpses might just reflect that both pathways can control cytoskeletal dynamics independently. Overexpression

experiments might represent a bypass, where overactivation of one pathway might compensate for disruption of a pathway that is indeed parallel. Furthermore, it has been observed that different types of cells (such as muscle vs. hypodermal as described above) utilize different genes during engulfment, and early and late engulfment events also might be regulated differently (Cabello *et al.*, 2010). Most studies of engulfment genes measure in real-time the duration of cell clearance of a few cells and also the total number of persistent cell corpses at a given time in development, but these two assays could be measuring intrinsically different phenomena (Cabello *et al.*, 2010). More studies must be done to determine how the many players of *C. elegans* engulfment interact and whether these interactions are global or specific to a cell-type or developmental period.

14. Cell corpse clearance in other organisms

Homologs of many of engulfment genes described above also control engulfment in other organisms. Although cell corpses in *C. elegans* are always engulfed by neighboring cells, cell corpses in flies and mammals are often engulfed by "professional" phagocytes, such as macrophages, microglia, or hemocytes (Shklover *et al.*, 2015). Thus, in addition to "eat me" signals such as phosphatidylserine, cell corpses must also send out "find me" signals to attract roaming phagocytes. These are thought to include fractalkine, lysophosphatidylcholine, sphingosine-1-phosphate, and nucleotides such as ATP and UTP (Hochreiter-Hufford and Ravichandran, 2013; Ravichandran, 2011). However, based on the expression patterns, concentration needed to attract

macrophages, or time-to-degradation of some of these signals, it is unclear which might act as effective long-range signals *in vivo*. Perhaps multiple signals can work together to attract professional phagocytes.

Once the apoptotic cell is found and recognized by a phagocyte, cytoskeletal changes occur in the engulfing cell to extend pseudopods via the homologs of the *C. elegans* engulfment genes. As in *C. elegans*, mammalian CrkII, ELMO1 and Dock180 (also known as Dock1) promote activation of Rac1; these genes are homologs of the *C. elegans* proteins CED-2, CED-12, CED-5, and CED-10, respectively (Ravichandran and Lorenz, 2007; Reddien and Horvitz, 2004). As might be the case in *C. elegans* with the integrin *ina-1*'s acting upstream of the *ced-2/5/10/12* pathway, mammalian integrins can mediate engulfment through the CrkII-Dock180-ELMO pathway (Albert *et al.*, 2000). The other major *C. elegans* engulfment pathway is conserved as well: CED-6 is homologous to GULP, CED-1 is homologous to CD91/LRP1 (Su *et al.*, 2002) or MEGF10 (Hamon *et al.*, 2006), and *ced-7* encodes an ABC transporter (Wu and Horvitz, 1998b). It has been shown that LRP1 can bind to GULP (Su *et al.*, 2002). MEGF10 can bind to the ABC transporter ABCA1, and these proteins might work together during engulfment (Hamon *et al.*, 2006). These genes are conserved in *Drosophila* as well, with CED-2, -5, -10, and -12 being homologous to DCrk, Myoblast City (MBC), DRac, Dced-12, respectively; and CED-1 and -6, homologous to Draper and D*ced-6* (Awasaki *et al.*, 2006; Callebaut *et al.*, 2003; Erickson *et al.*, 1997; Freeman *et al.*, 2003; Galletta *et al.*, 1999; Geisbrecht *et al.*, 2008; Luo *et al.*, 1994). In *Drosophila*, the E3 ubiquitin ligase complex containing Pallbearer, SkpA, and dCul1 is also involved in efficient engulfment (Silva *et al.*, 2007). This complex causes the

degradation of phosphorylated ribosomal protein S6, which leads to upregulation of Rac2 causing increased actin reorganization and efficient engulfment (Xiao *et al.*, 2015).

Similar to the *ced-2/5/10/12* genes, which promote proper distal tip cell migration in *C. elegans*, *crk*, *mbc, rac,* and *elmo* promote border cell migration in *Drosophila* (Murphy and Montell 1996; Duchek *et al*. 2001; Geisbrecht *et al*. 2008). During oogenesis, the border cells migrate from the anterior of the egg chamber posteriorly through the nurse cells until they reach the oocyte, then they migrate dorsally to the dorsal edge of the egg chamber (King and Koch 1963). Perturbation of *crk* (tested by RNAi), *mbc* (tested by loss-of-function mutation and RNAi), *rac* (tested by expression of dominant negative or constitutively active forms), or *elmo* (tested by RNAi) prevent the border cells from migrating fully or at all (Murphy and Montell 1996; Duchek *et al*. 2001; Geisbrecht *et al*. 2008). Based on mosaic analysis and tissue-specific knockdown experiments, these genes act cell-autonomously within the migrating cells to mediate cytoskeletal changes, such as the accumulation of F-actin, in response to extracellular signals (Duchek *et al*. 2001; Geisbrecht *et al*. 2008). Heat-shock induced expression of mutant forms of *rac* was used to demonstrate that *rac* is required for both initiation of and completion of border cell migration (Murphy and Montell 1996). Such analysis has not been done to discover the site of action of engulfment genes during the *C. elegans* distal tip cell migration, but engulfment genes might be similarly acting within the migrating distal tip cells.

A feature of mammalian apoptosis and corpse clearance is that it is typically immunologically silent. In contrast to necrosis, apoptosis is a way to remove cells

during development without causing inflammation (Bär, 1996; Nagata *et al.*, 2010). Macrophages that engulf apoptotic cells can produce anti-inflammatory cytokines, such as TGFβ or PGE2 (Fadok *et al.*, 1998b). One way that this can occur is through AMP signaling from apoptotic cells, which can signal through the A2a adenosine receptor to mediate an anti-inflammatory response (Yamaguchi *et al.*, 2014). *C. elegans* does not have an adaptive immune response, so these processes do not occur during engulfment in the worm.

Engulfment can promote programmed cell death

15. Engulfment promotes cell death in *C. elegans*

When the first *C. elegans* engulfment mutants were isolated, it was argued that cell deaths represented "suicides" instead of "murders," since most deaths still occurred without the engulfment process (Hedgecock *et al.*, 1983). Although engulfment is dispensable for cell death, these two processes are not unrelated. Engulfment can be observed to occur concomitantly with cell death, before the appearance of a fully-refractile cell corpse (Robertson and Thomson, 1982). Engulfment genes can also promote processes associated with cell death; *ced-1* and *ced-7* are required for the steps of DNA degradation that create TUNEL-positive ends, and engulfment is required for the completion of the DNA degradation process (Wu *et al.*, 2000).

There have been multiple studies of *C. elegans* that examined situations where cells were poised on the verge of death and found that engulfment genes promoted

death. Two of these studies were published together in 2001 and suggested that engulfment genes generally promote cell death to a small extent in many, if not all, cells (Hoeppner *et al.*, 2001; Reddien *et al.*, 2001). These two studies took different approaches.

Reddien and colleagues performed an enhancer screen using a weak *ced-3* background (2001). A weak *ced-3* mutation causes a small number of cells to stochastically survive; such a screen could potentially identify genes with minor roles in enhancing cell death that would have been too subtle to identify in a non-sensitized background. Surprisingly, they found that in a weak *ced-3* background, the loss of engulfment-gene function allowed extra cell survival. This enhancement occurred in a number of different cell types in the anterior pharynx, the postdeirid, and the ventral cord, suggesting that engulfment genes might act broadly to enhance cell death.

Hoeppner and colleagues monitored the first thirteen deaths of the AB lineage by four-dimensional microscopy (*i.e.*, a time course of z-stack images) to investigate the kinetics and morphological changes of cell death (2001). They found that in a weak *ced-3* background, one cell died and remained unengulfed (despite the intact engulfment pathway) and two appeared to initiate cell death but then recover. When they analyzed a weak *ced-3*; engulfment double-mutant, they found that more cells were able to recover and also many cells failed to initiate cell death at all. They proposed that engulfment genes might function in one of two ways to promote cell death: as a backup or as a positive feedback mechanism. Engulfment might function as a backup system for eliminating cells, which removes a dying cell before it could

recover. Alternatively, an engulfing cell might signal to a dying cell to further activate the programmed cell death pathway so that it reaches completion. Reddien and colleagues suggested that because some cells in weak *ced-3*; engulfment double-mutants fail to exhibit any signs of cell death at all, engulfment might play a role in the death process itself rather than passively preventing injured cells from recovering (2001).

Engulfment genes also play a role in a cytotoxic cell death in *C. elegans*. Gain-of-function mutations in *lin-24* or *lin-33* cause the hypodermal Pn.p blast cells to stochastically die inappropriately (Ferguson and Horvitz, 1985; Ferguson *et al.*, 1987). Pn.p cells in these mutant backgrounds increase in refractility, and then can recover to a normal morphology, recover with a small nucleus, or die (Galvin *et al.*, 2008). These deaths are morphologically and genetically distinguishable from apoptotic or necrotic deaths in *C. elegans* and seem to represent injury from cytotoxic molecular activity. Mutation of engulfment genes suppresses the death of these cells, especially mutation of the *ced-2/5/12* branch of the pathway (Ellis *et al.,* 1991; Galvin *et al.,* 2008; Wu *et al.,* 2001).

Together, these three studies established a role for engulfment in promoting cell death in both cytotoxic cell death and apoptotic cell death. Although almost all cells still die in a mutant deficient only for engulfment, the ability of the engulfment genes to promote death globally can be clearly seen in a sensitized background.

16. Engulfment can cause cell death in other organisms: phagoptosis

A form of cell death that is dependent on phagocytosis has been recently named phagoptosis (Brown and Neher, 2012). A death is considered phagoptotic if

it does not occur when phagocytosis is blocked. Although this type of cell death has only recently acquired a name, its existence has been recognized for much longer (Lagasse and Weissman, 1994). It is proposed that phagoptosis occurs when a viable cell displays or releases "eat-me" signals, such as phosphatidylserine or calreticulin, or stops displaying "don't-eat-me" signals, such as CD47 (Brown and Neher, 2012).

Under normal physiological conditions in mammals, phagoptosis might contribute to the death of erythrocytes and neutrophils (Brown and Neher, 2012). Erythrocytes display lower levels of the "don't-eat-me" signal CD47 as they age, and depletion of macrophages allows for these low-CD47, aged erythrocytes to accumulate (Khandelwal *et al.*, 2007). Although aged neutrophils normally display signs of apoptosis and are cleared by phagocytosis, apoptosis is not required for their clearance; bcl-2-overexpressing neutrophils are blocked from apoptosis but still are engulfed by macrophages and thus do not accumulate (Lagasse and Weissman, 1994). Loss of other don't-eat-me signals can also cause to apoptosis-independent cell loss. PAI-1 mutant neutrophils are engulfed and killed faster than PAI-1 expressing cells, and incubation with PAI-1 protein can suppress this cell killing (Park *et al.*, 2008). PAI-1 seems to suppress surface calreticulin levels, which might be how it mediates an affect on engulfment (Park *et al.*, 2008).

Cells can avoid death by phagoptosis by upregulating "don't-eat-me" signals. Of AML cancer cell lines that vary in CD47 expression, high CD47 lines survived better when injected into a host animal than low CD47 cell lines, and there appeared to be selective pressure towards maintaining high CD47 levels that was macrophage-dependent, presumably because phagoptosis continuously eliminated the low CD47

cells (Jaiswal *et al.*, 2009). *in vitro* macrophages preferentially engulf low-CD47 cells, and this preference is dependent on the function of the CD47 receptor, SIRPα (Jaiswal *et al.*, 2009). Chao *et al.* analyzed several cancer lines and surprisingly found that many have high surface levels of the "eat-me" signal calreticulin; this appears to be counterbalanced by high expression of CD47 (Chao *et al.*, 2010). Knocking down CD47 levels *in vitro* causes robust engulfment of these cells. Calreticulin is expressed in stressed or DNA-damaged cells, so high expression of CD47 might be selected for in order to protect these cells from being engulfed. Thus, blocking the action of CD47 would leave these cancer cells prone to engulfment. Indeed, anti-CD47 monoclonal antibodies have therapeutic potential and are being developed for use in the treatment of cancer (Chao *et al.*, 2011a, 2011b; Liu *et al.*, 2015; Majeti *et al.*, 2009; Willingham *et al.*, 2012).

Although phosphatidylserine exposure on the cell surface is often considered to be a marker of apoptosis, there are cases when living cells also externalize PS. For example, aging erythrocytes expose PS (Connor *et al.*, 1994). Certain treatments or cellular interactions can cause PS exposure, such as treatment by galectin in some leukocytes (Dias-Baruffi *et al.*, 2003), activation of cytotoxic T cells (Fischer *et al.*, 2006), maturation of macrophages (Callahan *et al.*, 2003), treatment with *N*-ethyl-maleimide (Kagan *et al.*, 2002), or interaction with macrophages (Jitkaew *et al.*, 2009). In many of these studies, the PS exposure is transient and the cells remain viable, but incubation with engulfing-competent cells can sometimes lead to apoptosis-independent cell loss (Dias-Baruffi *et al.*, 2003; Jitkaew *et al.*, 2009; Kagan *et al.*, 2002).

This effect is not limited to hematopoietic cells. Viable neurons can also be engulfed and killed by microglia during inflammation and normal development (Brown and Neher, 2012, 2014). The inflammatory agents lipopolysaccharide and lipoteichoic acid cause PS exposure and lead to of neuronal density loss that is dependent on the presence of microglia and the phosphatidylserine signaling pathway (Fricker *et al.*, 2012; Neher *et al.*, 2011). Ischemia also can cause a loss of neurons that is less severe in mutants of the phagocytic genes Mer receptor tyrosine kinase or milk fat globule EGF-like factor 8, presumably because these mutations block phagocytosis (Neher *et al.*, 2013). Low levels of amyloid-beta protein increases the phagocytic capacity of microglia as well as increasing exposure of phosphatidylserine on otherwise-viable neurons, resulting in neuronal loss *in vitro* that is dependent on the phosphatidylserine signaling pathway (Neniskyte *et al.*, 2011). Although the role of apoptosis genes has not been thoroughly investigated in these deaths, lipopolysaccharide-induced neuronal loss is not mitigated by caspase inhibitors or Bax mutation, suggesting that phagoptosis of viable neurons is an example of cellular "murder" rather than a variation of cell suicide (Fricker *et al.*, 2012). Phagoptosis may occur during normal developmental conditions as well. Microglia colonize proliferative zones of embryonic brains and engulf non-apoptotic neuronal precursor cells (Cunningham *et al.*, 2013). In a mouse model of retinal degeneration (retinitis pigmentosa), microglia were observed to engulf non-apoptotic cells, which did not have TUNEL staining or activated caspase-3, and inhibition of microglial phagocytosis reduced the progression of the disease (Zhao *et al.*, 2015).

Additionally, phagocytes, such as microglia, can promote apoptotic cell deaths. Purkinje cells in slices of the cerebellar cortex show signs of apoptosis (including caspase-3 activation and TUNEL staining), but selectively removing microglia results in increased numbers of viable neuronal precursors, suggesting that the microglia might be causing the deaths of the Purkinje cells (Marın-Teva *et al.*, 2004). Treatment with either a scavenger of superoxide ions or an inhibitor of NADPH oxidase also increased the number of viable cells in this study, demonstrating that microglia respiratory bursts might promote the deaths of neurons in a similar way that phagocytes kill microbes. However, it is unclear if engulfment *per se* is involved in this form cell death, or if the microglia act to induce cell suicide, as has been observed in other cases (Frade and Barde, 1998; Guadagno *et al.*, 2013).

17. The interplay between apoptotic and engulfment pathways is unclear: cell competition in *Drosophila*

In mosaic or chimeric animals, neighboring cells of different genotypes can have different relative fitness. These differences can cause certain cells to proliferate more than others, and this phenomenon is called “cell competition” (Morata and Ripoll, 1975). In the imaginal discs of *Drosophila* wild-type cells proliferate faster than cells heterozygous for the Minute mutation (genotype M/+), which undergo apoptosis when adjacent to wild-type cells (Moreno *et al.*, 2002). This death is required for cell competition, as expression of the caspase inhibitor p35 prevented the out-competition of the M/+ cells (Moreno *et al.*, 2002). The dying “loser” cells contained active caspase-3 and were engulfed by neighboring “winner” wild-type

cells (Li and Baker, 2007). To allow corpses to accumulate for easier quantification, Li and Baker attempted to eliminate engulfment by mutation or RNAi treatment of the *ced-1* homolog *draper (drpr)*, mutation of the actin regulator *wasp*, or mutation of the putative phosphatidylserine receptor *psr* (2007). Surprisingly, instead of allowing corpses to accumulate, eliminating engulfment significantly reduced cell competition and cell death: M/+ cells represented a larger percentage of overall area, and the number of M/+ corpses was reduced two-fold or more. Conversely, over-activation of *wasp* caused increased death of wild-type neighboring cells, suggesting that engulfment genes can promote death of otherwise viable cells. These findings led Li and Baker to claim that engulfment is required for cell competition, even though loss of engulfment does not completely eliminate this cell death (2007). Another lab found that the engulfment genes *mbc* and *Dced-12* (homologs of *C. elegans ced-5* and *ced-12*, respectively) were similarly required for the elimination of mutant clones in the imaginal disc that had mutation of tumor suppressor genes (*scrib* or *dlg*) (Ohsawa *et al.*, 2011).

Some of these findings have been called into question. The Baker lab also performed an unbiased genetic screen for mutations that prevent cell competition, but they did not report isolating any mutations of engulfment genes as would be expected (Tyler *et al.*, 2007). Martín *et al.* found that *drpr* mutation did reduce the number of apoptotic corpses at the borders of M/+ and wild-type clones; however they did not find that *drpr* mutation affected the size of the clones (Martín *et al.*, 2009). Lolo *et al.* examined the effect of *wasp, drpr,* or *psr* mutation using both the Minute competition assay and a Myc supercompetition assay and found that cell

competition and cell death still occurred in all mutant backgrounds (2012). Cell corpses were extruded basally and engulfed by both neighboring winner cells and by circulating hemocytes (Lolo *et al.*, 2012). Li and Baker did observe basal accumulation of apoptotic material, but they did not determine whether it was engulfed (Li and Baker, 2007). Lolo and colleagues suggest that the discrepancy between these two studies might be the result of Li and Baker failing to take into account the basal apoptotic material or counting circulating hemocytes as living cells (Lolo *et al.*, 2012). More replication is necessary to determine the role of engulfment genes in promoting cell death in cases of cell competition. A recent study argues that cell-cell intercalation, driven by differences in tension at cell interfaces, is required for cell competition (Levayer *et al.*, 2015). Thus, it is possible that engulfment genes, which act upstream of the cytoskeleton, might have nonspecific effects on cell tension that could affect the efficiency of cell competition.

18. One cell death in *C. elegans* is engulfment-dependent

There were two male-specific cells in *C. elegans* that seemed to be killed instead of undergoing suicides (Sulston *et al.,* 1980). Specifically, the linker cell and the one of the pair of B.alapaav and B.arapaav undergo programmed cell death, and these deaths were reported to fail when the engulfing cell was removed by laser ablation or when engulfment function was blocked by mutation (Hedgecock *et al.*, 1983; Sulston *et al.*, 1980). More detailed studies of the linker cell death demonstrated that it is not actually dependent on engulfment by U.l/rp (Abraham *et al.*, 2007). It also can die even if it becomes mis-localized away from U.l/rp, and the death can precede engulfment (Abraham *et al.*, 2007; Kinet and Shaham, 2014).

Interestingly, U.l/rp are able to readily engulf the linker cell even when the typical engulfment genes are mutated, suggesting that the linker cell might be engulfed using a completely separate genetic pathway than what is used by apoptotic and necrotic corpses (Abraham *et al.*, 2007). The linker cell also dies by a non-apoptotic mechanism (Abraham *et al.*, 2007; Blum *et al.*, 2012). While the linker cell remains unique and a topic of active research, it does not represent an engulfment-dependent murder. The engulfment-dependence of the B.al/rapaav cell death has been replicated (Reddien *et al.*, 2001), and the genetic requirements of this unique cell death are described in further depth in Chapter 2 of this thesis.

Acknowledgments

Thank you to Kaitlin Driscoll and my thesis committee for comments on this chapter.

Figure 1: **An image of the developing *C. elegans* male tail taken using Nomarski optics.** Many nuclei are visible, including hypodermal nuclei (with "fried egg" morphology, *i.e.* a recessed nucleus with a well-defined, raised nucleolus) and neurons (with "pepperoni" morphology, *i.e.* a speckled nucleus without a well-defined nucleolus). Three cell corpses are visible, which are round and refractile. The larger of the three corpses just recently died, so it is not yet completely smooth in appearance. This is the same image that was used in Chapter 2, Figure 2A. Anterior is to the left, dorsal is up. Scale bar: 10 μm.

Figure 1

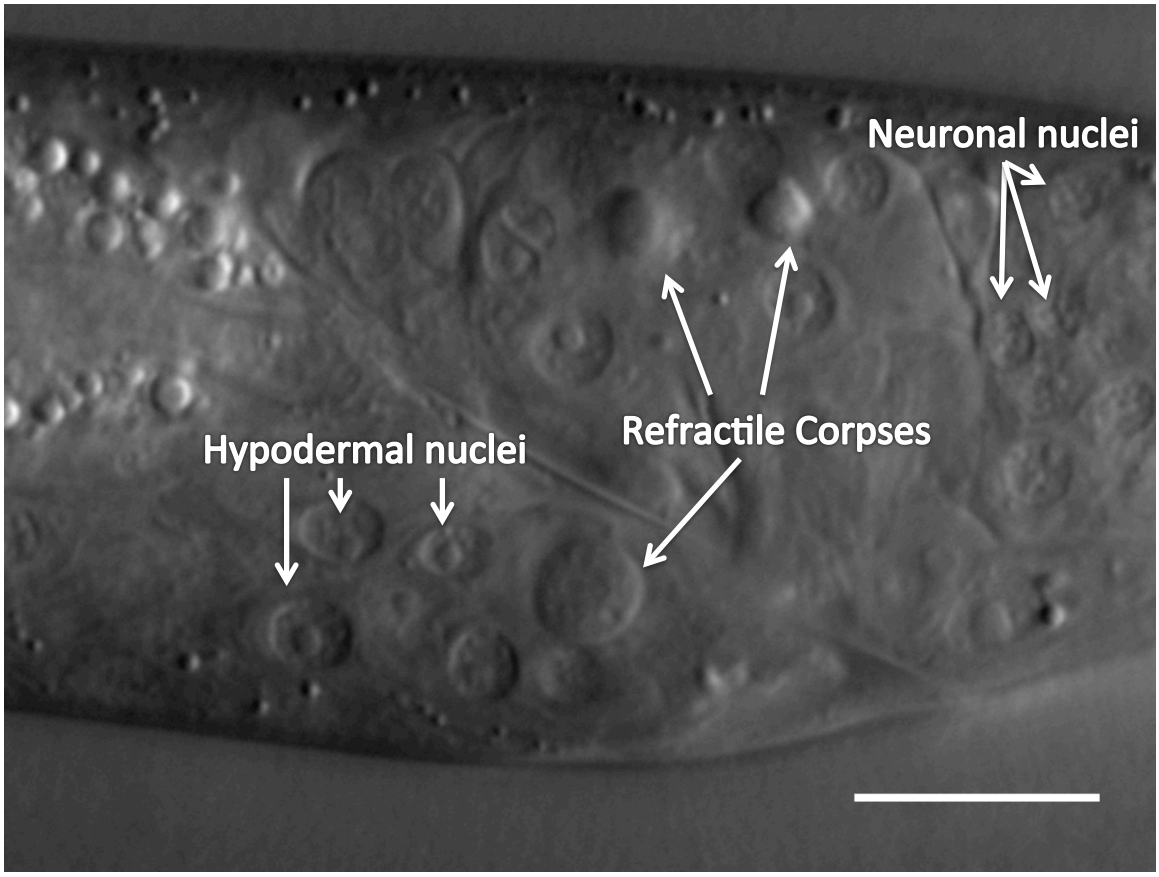


Figure 2: **The intrinsic apoptosis pathways in *C. elegans*, mammals, and *D. melanogaster*.** The *C. elegans* and *D. melanogaster* apoptotic pathways each have elements that are similar to the mammalian apoptotic pathway. These diagrams are simplifications designed to highlight the structural similarities and differences between these organisms; for more detail, see text. Red: pro-apoptotic BH3-only proteins of the Bcl-2 family. Orange: anti-apoptotic Bcl-2 family proteins. Yellow: pro-apoptotic Bcl-2 family proteins. Green: conserved apoptosome proteins. Blue: inhibitors-of-apoptosis proteins. Purple: caspases. Brown: IAP antagonists. Boxes represent key targets of regulation that, when activated, initiate cell death.

Figure 2

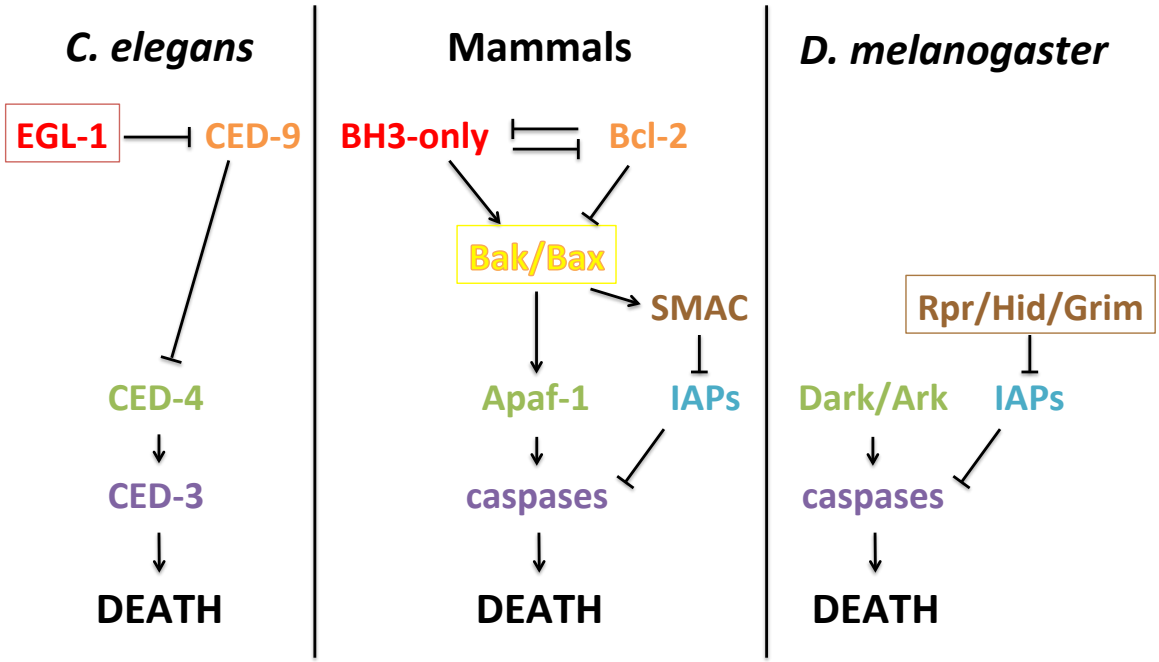
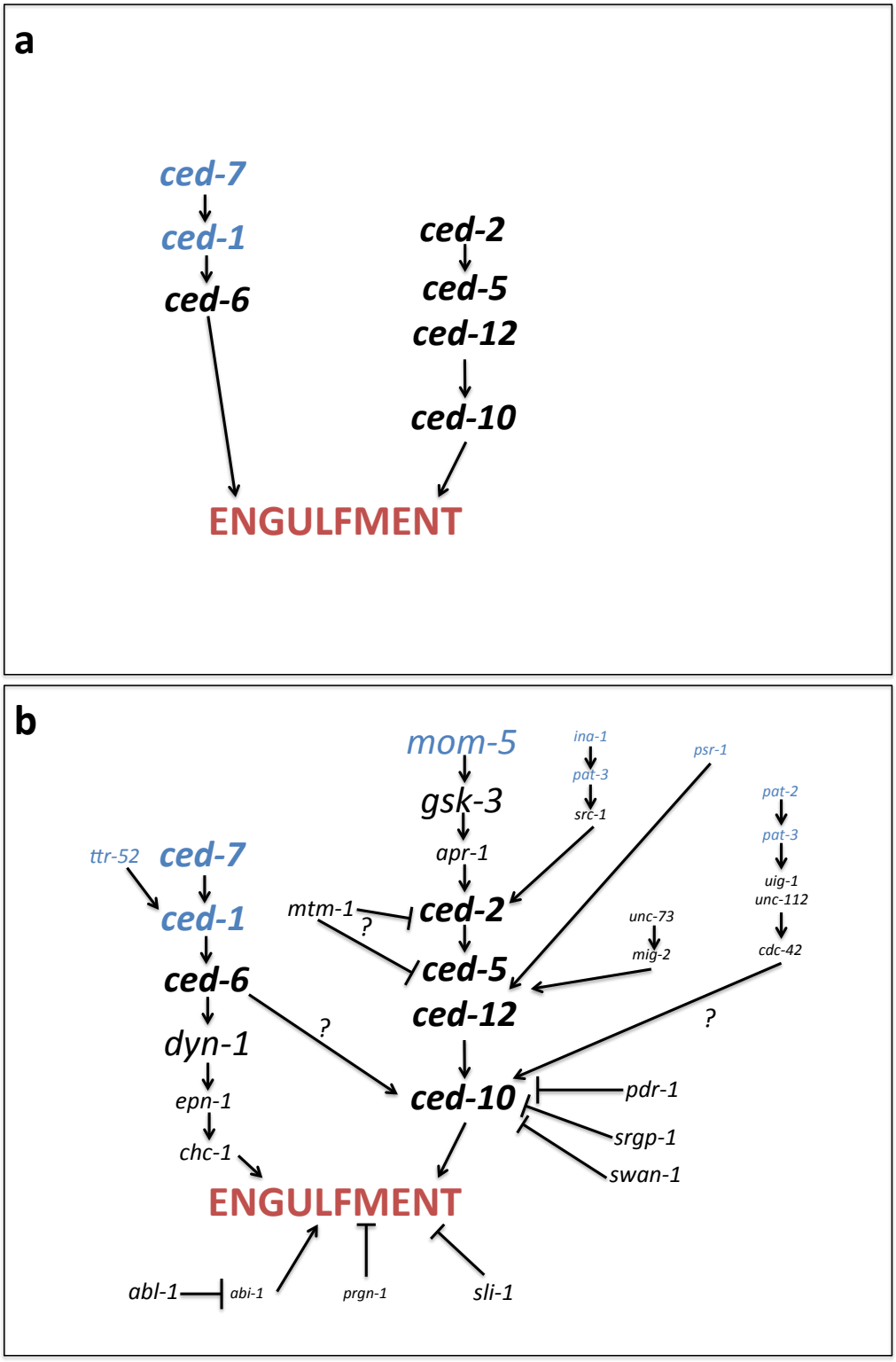

C. elegans
EGL-1
CED-9
CED-4
CED-3
DEATH
Mammals
BH3-only
Bcl-2
Bak/Bax
SMAC
Apaf-1
IAPs
caspases
DEATH
D. melanogaster
Rpr/Hid/Grim
Dark/Ark
IAPs
caspases
DEATH

Figure 3: **A schematic of the genetic control of engulfment in *C. elegans*. a**, The classical engulfment genes (*i.e*., isolated before the end of 2000) act in two parallel, partially redundant pathways. Blue text, genes that are thought to be involved in recognition of cell corpses. **b**, The classical engulfment pathways (bold text) have been supplemented by the identification and characterization of many more genes that affect engulfment, including negative regulators. Blue text, genes that are thought to be involved in recognition of cell corpses. Text size represents the approximate relative contribution of each gene, *i.e.* large text denotes that a mutant causes engulfment defects similar in strength to strong mutants of the classical engulfment genes, medium-sized text denotes that a mutant causes engulfment defects approximately half as strong as strong mutants of the classical engulfment genes, and small text denotes that mutation causes a weak engulfment phenotype, which may only be visible in a sensitized background. Note that if a null allele is not available for a gene, the strength of its contribution might be underestimated. In the case of negative regulators of engulfment, medium-sized text denotes that a mutant partially suppresses the engulfment-defective phenotype of classical engulfment gene and small text denotes that mutation does not suppress the engulfment-defective phenotype of strong engulfment mutants.

For more information about the function of each of these genes and the evidence of their interactions with other engulfment genes, see text.

Figure 3


a
ced-7
ced-1
ced-6
ced-2
ced-5
ced-12
ced-10
ENGULFMENT
b
mom-5
gsk-3
apr-1
ina-1
pat-3
src-1
psr-1
pat-2
pat-3
uig-1
unc-112
cdc-42
ttr-52
ced-7
ced-1
ced-6
dyn-1
epn-1
chc-1
mtm-1
?
ced-2
ced-5
unc-73
mig-2
ced-12
?
?
ced-10
pdr-1
srgp-1
swan-1
ENGULFMENT
abl-1
abi-1
prgn-1
sli-1

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Chapter 2

An Assisted-Suicide Programmed Cell Death

in *Caenorhabditis elegans*

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Summary

Programmed cell deaths in the nematode *Caenorhabditis elegans* are generally considered suicides. Dying cells are engulfed by neighboring cells in a process of phagocytosis. To better understand the interaction between the engulfment and death processes, we analyzed the B.al/rapaav cell death, which has been previously described as engulfment-dependent and hence as a possible murder. We found that the caspase-mediated suicide pathway initiates the cell-death process but is insufficient to cause B.al/rapaav death without the subsequent assistance of engulfment. When the engulfing cell P12.pa is absent, other typically non-phagocytic cells can display cryptic engulfment potential and facilitate this death. We term this death an "assisted suicide." We propose that assisted suicides likely occur in other organisms. The study of assisted suicides might provide insight into non-cell autonomous factors of cell death and suggest ways to modulate the sensitivity of cells to apoptosis and treat diseases associated with the dysregulation of apoptosis.

Introduction

Programmed cell death, often referred to as apoptosis, is an evolutionarily conserved process that plays critical roles in normal animal development and tissue homeostasis; dysregulation of programmed cell death can cause disorders as diverse as cancer, autoimmune disease and retinal degeneration (Thompson 1995; Baehrecke 2002). The genetic pathway that controls programmed cell death in *C. elegans* is known and evolutionarily conserved. Both pro-death and pro-survival

proteins are likely present in most if not all cells (Shaham and Horvitz 1996). The decision of a cell to die is generally made at the level of the transcriptional control of the pro-apoptotic gene *egl-1* (Conradt and Horvitz 1999). When the EGL-1 BH3-only protein is produced, it disrupts the cell-protective interaction between the anti-apoptotic CED-9 Bcl-2-family protein and the CED-4 Apaf-1-like protein, releasing CED-4 to activate the pro-apoptotic caspase CED-3 (Shaham and Horvitz 1996; Wu *et al.* 1997; Conradt and Horvitz 1998; del Peso *et al.* 1998; Yang *et al.* 1998; Conradt and Horvitz 1999;). These genes function cell-autonomously in cell death (Yuan and Horvitz 1990; Shaham and Horvitz 1996), and for this reason *C. elegans* cell deaths generally have been considered to be suicides.

Each dying cell is engulfed by a neighboring cell. In mutants deficient in the engulfment process, the vast majority of cells still undergo programmed cell death, but the cell corpses persist unengulfed and undegraded (Hedgecock *et al.* 1983; Ellis *et al.* 1991; Reddien *et al.* 2001). Only a very small number of cells stochastically fail to die in engulfment-defective mutants (Reddien *et al.* 2001). Thus, engulfment is not generally required for the death process. Engulfment can promote the deaths of sensitized cells (*e.g.*, in mutants with reduced *ced-3* function), possibly by preventing injured cells from recovering and surviving (Reddien *et al.* 2001; Hoeppner *et al.* 2001; Neukomm *et al.* 2011). By contrast, there is one cell death in *C. elegans* that has been speculated to be a murder, based on the observation that if the engulfing cell is killed using laser microsurgery, the cell death can be prevented. Specifically, the two left-right homologous cells B.alapaav and B.arapaav in the tail of the third-larval stage male constitute a developmental equivalence group (Sulston

and White 1980; Sulston *et al.* 1980): either can survive (the primary fate), while the other undergoes programmed cell death and is engulfed by a neighboring cell, P12.pa (the secondary fate) (Figure 1A-C). This death occurs during the fourth larval stage (Sulston and Horvitz 1977).

Two lines of evidence suggest that this programmed cell death is not a normal suicide. First, both B.alapaav and B.arapaav can survive in animals in which the engulfing cell P12.pa has been ablated with a laser microbeam (Sulston *et al.* 1980). Second, the B.al/rapaav death does not occur in mutants defective in the engulfment genes *ced-1* or *ced-2* (Hedgecock *et al.* 1983). (We use "B.al/rapaav" to refer to the homolog that dies or that is fated to die [see below], which can be either B.alapaav or B.arapaav. We use "the B.al/rapaav homolog" to refer to the homolog that is fated to survive.) These observations suggested that the B.al/rapaav death is dependent on P12.pa and that this death might be a murder mediated by the engulfment process. However, it was later observed that the B.al/rapaav death also fails in mutants defective in the caspase *ced-3*, leading to the suggestion that this death might be an induced suicide initiated by P12.pa engulfment or by another function of the engulfment genes (Reddien *et al.* 2001). We sought to characterize this unusual programmed cell death to further understand the interaction between the cell autonomously-acting cell-death genes and the cell non-autonomous process of engulfment.

Results

The B.al/rapaav death is caspase- and engulfment gene-dependent

To confirm and extend the observations that the B.al/rapaav death is dependent on the engulfment genes *ced-1* and *ced-2* (Hedgecock *et al.* 1983) and the caspase *ced-3* (Reddien *et al.* 2001), we assayed the B.al/rapaav death in a variety of cell-death mutant backgrounds. Specifically, we scored the presence of a cell that expressed a reporter specific for the dying B.al/rapaav, $P_{egl\text{-}1}$*::4xNLS::gfp* (see below), in a position consistent with that of an undead B.al/rapaav in late fourth larval stage animals and interpreted such a cell as one that had failed to undergo programmed cell death. In this way, we confirmed that the B.al/rapaav cell death is dependent on both suicide and engulfment pathways. Specifically, strong loss-of-function alleles of the pro-apoptotic genes *egl-1, ced-4* or *ced-3* or a gain-of-function allele of the anti-apoptotic gene *ced-9* almost completely blocked the B.al/rapaav death (Figure 1D). Strong loss-of-function alleles of any of the major engulfment genes were also sufficient to prevent the B.al/rapaav death (Figure 1D). While loss of engulfment gene function can weakly contribute to cell survival, the effect is smaller than that of weak reduction-of-function mutations in suicide genes (Reddien *et al.* 2001). By contrast, weak reduction-of-function mutations in the pro-apoptotic genes *ced-3* or *ced-4* had smaller effects on B.al/rapaav survival than did loss of engulfment gene function (Figure 1D). This finding indicates that the B.al/rapaav death is specifically dependent on the function of engulfment genes rather than highly sensitive to any slight perturbation to the cell-death pathway. A loss-of-function mutation in *unc-108* Rab2, a gene important for phagosome maturation and cell-corpse degradation after engulfment (Mangahas *et al.* 2008), did not

significantly block the B.al/rapaav death, suggesting that the engulfment process and not downstream degradation processes are important for the B.al/rapaav death.

The dying B.al/rapaav expresses cell-death genes

The cell-death genes in *C. elegans* act cell-autonomously to specify and cause cell death (Yuan and Horvitz 1990; Shaham and Horvitz 1996). To examine if the presumptive cell-death suicide genes indeed act in the dying B.al/rapaav, we used a transcriptional reporter for *egl-1*, the most upstream gene in the core cell-death pathway; *egl-1* is transcriptionally regulated to drive programmed cell death (Conradt and Horvitz 1999). The *egl-1* reporter was expressed strongly in the B.al/rapaav cell fated to die and not at all or only weakly in the B.al/rapaav homolog, which is fated to live (Figure 2A,B). All B.al/rapaav corpses in fourth-larval stage wild-type animals were GFP-positive (n = 53), confirming that the GFP expression is associated with the secondary fate of cell death.

We used single-molecule fluorescent *in situ* hybridization (smFISH) to determine whether endogenous cell-death genes, like the $P_{egl\text{-}1}$*::4xNLS::gfp* reporter, are transcribed in the dying B.al/rapaav. We usually detected *egl-1* mRNA expression in B.al/rapaav and only rarely in the B.al/rapaav homolog, consistent with the expression pattern of $P_{egl\text{-}1}$*::4xNLS::gfp* (Figure 2C-E). We detected low levels of *ced-9* and *ced-4* transcripts broadly (Figure 3). *ced-3* transcripts were visible in only a subset of cells, usually including B.al/rapaav, and only rarely in the B.al/rapaav homolog (Figure 2C-E). Primary- and secondary-fated cells were

identified based on nuclear position and morphology (the secondary-fated B.al/rapaav cell nucleus appears condensed after staining DNA with DAPI to visualize nuclei and is closer to P12.pa). Because the B.al/rapaav cell death is dependent on suicide genes that are expressed in the dying B.al/rapaav and not in nearby cells, the B.al/rapaav death is likely a form of cell suicide rather than a murder.

Engulfment genes do not induce the caspase-mediated suicide pathway

As in wild-type animals, $P_{egl\text{-}1}$*::4xNLS::gfp* was expressed in the secondary B.al/rapaav even in cell-death and engulfment mutants (Figure 4A,B). Similarly, endogenous transcripts of the cell-death genes *egl-1* and *ced-3* were generally present in one, but not both, of B.alapaav and B.arapaav in the engulfment-defective double mutant, *ced-12 ced-1* (Figure 4C-G). These data indicate that the induction of suicide gene expression in B.al/rapaav requires neither engulfment nor signals transduced via the engulfment pathway.

The dying B.al/rapaav exhibited morphological changes as visualized with Nomarski differential interference microscopy (DIC) similar to those of other dying cells (Robertson and Thomson 1982; Hoeppner *et al.* 2001). At the time of their generation, B.alapaav and B.arapaav are indistinguishable using DIC and appear similar to normal healthy cells (Figure 5A). Later, one (B.al/rapaav) becomes round and condensed with partial cytoplasmic refractility (Figure 5B) before becoming a highly refractile corpse (Figure 5C). The other (the B.al/rapaav homolog) remains non-refractile and looks healthy and normal.

While mutations in either the suicide pathway or the engulfment pathway were sufficient to block B.al/rapaav from acquiring the highly refractile appearance characteristic of programmed cell death, we discovered that the morphology of B.al/rapaav as viewed with DIC optics was different between these two classes of mutants: in the mid-fourth larval stage, B.al/rapaav was a highly-refractile corpse in most wild-type animals (Figure 5D); the undead B.al/rapaav in engulfment mutants generally was round with a refractile cytoplasm and non-refractile nucleus (Figure 5E,G); and the undead B.al/rapaav in *ced-3* and other suicide mutants generally was non-refractile, similar to other living cells, including the B.al/rapaav homolog (Figure 5F,H). The morphology of the undead B.al/rapaav in engulfment mutants was indistinguishable from that of the dying B.al/rapaav at an earlier stage of the cell-death process. We interpret this morphology to be that of a cell in which caspases have been at least partially activated, as this morphology is *ced-3* dependent. These results further show that the engulfment genes are not required for the initiation of the cell-death process but rather are required in a later process.

Although B.al/rapaav shows signs of initiating the cell-death pathway in engulfment mutants, the B.al/rapaav death is not completed in engulfment mutants. Similar to living cells the undead B.al/rapaav cells of engulfment mutants retained nuclear localization of P_{egl-1}::4xNLS::GFP, membrane localization of the cytoplasmic membrane marker P_{evl-20}::mCherry::PH (see below) and diffuse chromatin as revealed by DAPI staining (Figure 6A-C,G-I,J,L); by contrast, in fully-refractile B.al/rapaav corpses P_{egl-1}::4xNLS::GFP and P_{evl-20}::mCherry::PH were distributed throughout the cell, and chromatin after DAPI staining appeared condensed (Figure

6D-F,K). The distribution of P_{egl-1}::4xNLS::GFP throughout the cell corpse likely reflects nuclear disruption and/or inactivation of nuclear transport; nuclear disruption and chromatin condensation are two hallmarks of apoptotic cell death (Kerr *et al.* 1972). Additionally, during time-course observations, we occasionally saw fluctuations in the level of B.al/rapaav cytoplasmic refractility in engulfment mutants, as has been previously reported for other cells (Reddien *et al.* 2001). B.al/rapaav, like these other cells with partial cytoplasmic refractility, cannot be dead -- it can recover. Thus, *ced-3* activation does not inevitably lead to the death of B.al/rapaav.

The engulfing cell P12.pa is not required for the B.al/rapaav death

It has been reported that the engulfing cell P12.pa is required for the B.al/rapaav cell death (Sulston *et al.* 1980). To examine the role of P12.pa in the B.al/rapaav cell death, we ablated P12.pa or its precursor P12.p early in development, about 15-20 hours prior to the generation of B.alapaav and B.arapaav, and looked for signs that B.al/rapaav had initiated the cell-death program. In the absence of P12.pa, B.alapaav and B.arapaav adopted normal primary and secondary fates and one initiated the cell-death process, as evidenced by *egl-1* reporter gene expression and by the positions and morphologies of the two cells (Figure 7A-C). Thus, P12.pa is not required for B.alapaav and B.arapaav to differentially adopt the primary and secondary fates or to initiate the death process in the secondary B.al/rapaav.

To our surprise, we found that B.al/rapaav died in many of the animals that lacked P12.pa (Figure 7A), contrary to the previous report (Sulston *et al.* 1980). Thus, although the B.al/rapaav death is almost completely dependent on engulfment genes, it is only partially dependent on the presence of the normal engulfing cell. To reconcile this difference, we postulated that other cells might engulf B.al/rapaav in the absence of P12.pa. We designed a fusion protein of mCherry with the pleckstrin homology domain, which binds to lipids in the plasma membrane (Saraste and Hyvönen 1995), and expressed this construct using a promoter from the *evl-20* gene (Antoshechkin and Han 2002), which is widely expressed throughout the animal, including in P12.pa, B.alapaav and B.arapaav (Figure 7D,E). This construct highlights cell membranes and allowed us to visually determine if a corpse is internalized by another cell. We ablated P12.pa in this strain and found again that B.al/rapaav died in many of the animals. In animals in which B.al/rapaav survived, the undead B.al/rapaav was not engulfed (Figure 7D,F). B.al/rapaav died in 16 P12.pa-ablated animals, and in 14 of these 16 cases the B.al/rapaav corpse was engulfed by a neighboring cell (Figure 7E,F). We conclude that the discrepancy between the essentially complete dependence on engulfment genes and the weaker dependence on the engulfing cell P12.pa is a consequence of the compensatory ability of other cells to engulf B.al/rapaav in the absence of P12.pa.

In nine animals, the engulfing cell was the surviving primary B.al/rapaav homolog and in five animals it was a more lateral cell. The identities of the other engulfing cells are uncertain. Based on position, likely candidates are K.a or the left rectal gland cell, B.alapaad and F.lvv. The B.al/rapaav homolog, K.a, the rectal gland

cells, B.al/rapaad and F.l/rvv are cells that do not normally engulf corpses, since the only nearby dying cell is B.al/rapaav, which is engulfed by P12.pa (Sulston *et al.* 1980, and see below). These findings demonstrate that other cells are competent to engulf B.al/rapaav in the absence of P12.pa and that even cells that do not normally engulf cell corpses can have a cryptic ability to recognize and engulf dying cells to promote cell death. In short, B.al/rapaav likely becomes fated to die and begins to die cell-autonomously but generally requires engulfment by P12.pa or another neighboring cell to fully execute the death process. The death of B.al/rapaav thus does not seem to be an induced suicide, the process proposed by Reddien *et al.* (2001).

Engulfment by P12.pa precedes the B.al/rapaav death

If engulfment is required for the B.al/rapaav death, B.al/rapaav might be engulfed early in the cell-death process, before it becomes a fully refractile corpse. To test this hypothesis, we imaged B.al/rapaav in animals with cell membranes labeled by $P_{evl\text{-}20}$::mCherry::PH. We characterized the morphology of each B.al/rapaav as either non-refractile, round with cytoplasmic refractility (cell-death initiated) or fully refractile (dead). Then we imaged $P_{evl\text{-}20}$::mCherry::PH to determine the boundaries of B.al/rapaav and P12.pa. We found that B.al/rapaav corpses were always (19/19) internalized by P12.pa, cell-death initiated cells were usually (14/16) internalized by P12.pa, and cells with no sign of cell death initiation were never (0/10) internalized (Figure 8A). These data indicate that P12.pa engulfs B.al/rapaav in the early stages of its death, but probably not before the cell death

has already been initiated. These findings are consistent with our observations that the suicide pathway acts before the engulfment pathway. We also confirmed that the identity of the engulfing cell was always P12.pa in intact animals (*n* = 33): other cells engulf B.al/rapaav only if P12.pa is absent.

We confirmed that engulfment can precede cell death using electron microscopy. We observed a dying B.arapaav cell using DIC microscopy and fixed and stained the animal at the stage at which the B.arapaav cell was round with cytoplasmic refractility (Figure 8B). B.arapaav had already been internalized by P12.pa but ultrastructurally lacked signs of cell death, such as nuclear envelope dilation or crenulation, extensive chromatin condensation or membrane whorling (Figure 8C) (Robertson and Thomson 1982).

It is conceivable that engulfment genes act to promote the B.al/rapaav cell death by cell-killing activities independent of their roles in engulfment *per se*. The weak loss-of-function allele *ced-10(n1993rf)* stochastically prevents the B.al/rapaav cell death in only some animals (Figure 1D). We hypothesized that if *ced-10* promoted the B.al/rapaav death through a cell-killing activity independent of its role in engulfment, these two functions might stochastically fail in different animals. For example, B.al/rapaav might be killed without being engulfed or engulfed without being killed. However, if the cell-killing activity of *ced-10* were mediated through engulfment, B.al/rapaav would not be killed unless it were engulfed. We found that B.al/rapaav corpses in *ced-10(n1993rf)* were always engulfed by P12.pa (*n* = 13). Undead B.al/rapaav cells were sometimes unengulfed and sometimes engulfed (Figure 8D). These data are inconsistent with the hypothesis that

engulfment and cell-killing are independent events (P = 0.0075). We conclude that engulfment precedes the B.al/rapaav death and that the killing activity of *ced-10* is mediated through its engulfment function.

Discussion

Like most other cell deaths in *C. elegans*, the B.al/rapaav death requires the canonical suicide genes, and these genes are expressed in the cell that dies. But while the activity of the suicide program is sufficient to kill most cells (Figure 9A), it is insufficient to kill B.al/rapaav, which appears injured but intact without engulfment. Thus, engulfment is dispensable for other cell deaths but is required for the B.al/rapaav death. This death is unlikely to be a murder (Figure 9B), as it requires suicide genes, or an induced suicide (Figure 9C), as neither the engulfing cell P12.pa nor the engulfment genes are required for the initiation of the cell-death process. We conclude that the B.al/rapaav death is instead an assisted suicide (Figure 9D), since engulfment occurs early in the B.al/rapaav death process and is necessary to facilitate the suicide process and cause cell death.

Are there other assisted suicides? Cell deaths in *C. elegans* exist along a spectrum of sensitivities to engulfment. For example, in the anterior pharynx, postdeirid or ventral cord cells that are fated to die survive only 0-7% of the time in a *ced-1* engulfment mutant (Reddien *et al.* 2001), making B.al/rapaav, which survives 96% of the time (Figure 1D), a clear outlier. The B.al/rapaav death is the only death in *C. elegans* known to be so dependent on engulfment. However, comprehensive studies have not been done. The B.al/rapaav death was identified as

possibly engulfment-dependent and further studied in engulfment mutants based on its major dependence on the engulfing cell P12.pa (Sulston *et al.* 1980). B.al/rapaav is always engulfed by P12.pa in intact animals. By contrast, Hoeppner *et al.* (2001) found that for nine of 13 embryonic cell deaths studied the identity of the engulfing cell varied among animals. Additionally, we have observed that in the case of B.al/rapaav cells that do not normally display engulfment activity can have cryptic engulfment ability and engulf the dying cell if the normal engulfing cell is absent. In such cases, ablation of an engulfing cell would not prevent cell death, since a secondary engulfing cell would assume the engulfment function. More comprehensive analysis using engulfment-defective mutants rather than laser-ablation experiments could reveal more instances of assisted suicide in *C. elegans*.

How might engulfment promote the death process? It has recently been reported that engulfment genes promote the death of the sister cell of the *C. elegans* NSM neuron by affecting its precursor cell (Chakraborty *et al.* 2015). Specifically, in the precursor cell that generates the NSM neuron and its sister cell, which dies, there is a higher level of CED-3 activity in the region that will form the dying NSM sister. The engulfment genes *ced-1* and *ced-2* are required for the formation of this gradient, suggesting that engulfment can promote the cell-death process at least in part by causing cells to differentially inherit apoptotic potential. This mechanism is unlikely to be the way in which engulfment causes the B.al/rapaav death. First, B.alapaav and B.arapaav are not sister cells. Second, at the time of their generation neither is committed to dying (Sulston and White 1980), so that even if B.alapaav and B.arapaav inherit more pro-apoptotic potential than their sister cells, that

potential is not sufficient to cause cell death. Third, engulfment genes are not required for the difference in levels of apoptotic activity in B.al/rapaav and the B.al/rapaav homolog: we have shown that the dying cell B.al/rapaav up-regulates cell-death genes and displays morphological changes characteristic of cell-death initiation while the surviving B.al/rapaav homolog does not do so, and neither of these differences is engulfment-dependent.

Engulfment- or phagocyte-dependent cell deaths have been described in other organisms. For example, phagoptosis of stressed-but-viable neurons in mammals (Brown and Neher 2014) and entosis of weaker-but-viable cells in tumors (Sun *et al.* 2014) are engulfment-dependent mechanisms of cell killing. Tumor cells can evade phagocytosis by upregulating the "don't eat me" signal CD47, and this evasion can be abrogated with anti-CD47 antibodies, which allow tumor cells to be engulfed and killed (Chao *et al.* 2011). However, these deaths appear to be caspase-independent murders rather than caspase-dependent assisted suicides. It was reported that both engulfment and caspase genes are necessary for cell competition in *Drosophila* , a phenomenon in which fitter cells can outcompete and cause the death of neighboring, less fit cells (Li and Baker 2007), but more recent studies suggest that these deaths might not be engulfment-dependent (Lolo *et al.* 2012). Purkinje cells in slices of the mouse cerebellar cortex show signs of apoptosis (including caspase-3 activation and TUNEL staining) that is dependent on microglia, which are engulfing cells (Marín-Teva *et al.* 2004). However, it is unclear if engulfment is involved or if the microglia act to induce cell suicide by releasing

reactive oxygen species. Suicides induced by microglia have been observed in other cases (Frade and Barde 1998; Guadagno *et al.* 2013).

Do assisted suicides exist in other species? It might be difficult to identify such assisted suicides, since suicide-initiated stalled cells in *C. elegans* engulfment mutants appear morphologically similar to normal cell suicides in progress. In *C. elegans* it is possible to identify stalled cells as failed deaths because it is known when and where all cell deaths normally occur. It is likely that findings from future studies of assisted suicide in *C. elegans* will provide insights into the cell non-autonomous factors involved in engulfment-dependent deaths of other species. Better understanding of assisted suicides in *C. elegans* could also lead to the identification and study of assisted suicides in other organisms. Given that engulfment-mediated cell deaths likely act in the progression of various human diseases, such as the loss of neurons by phagoptosis in neurodegenerative disorders (Brown and Neher 2014) and the removal of cancer cells through engulfment or entosis (Chao *et al.* 2011; Sun *et al.* 2014), further studies of assisted suicides might provide insights of therapeutic importance.

Methods

Strains and Genetics.

C. elegans was maintained on nematode growth medium (NGM) Petri plates at 20°C. *him-8(e1489), him-5(e1490)* and *him-5(e1467ts)* were used as the wild-type

backgrounds, because mutations in *him-8* or *him-5* cause hermaphrodites to generate a high incidence of males, which facilitated study of the male-specific cell B.al/rapaav. The following mutations, integrations, and extrachromosomal arrays were used: LGI: *unc-108(nu415), ced-12(n3261), ced-1(e1735).* LGII: *nIs343* [P_{egl-1}*::4xNLS::gfp, lin-15AB(+)*]. LGIII: *ced-4(n3332lf), ced-4(n3195rf), dpy-17(e164), lon-1(e1820), lon-1(e185), ced-6(n1813), unc-32(e189), ced-7(n1892), ced-9(n1950gf)*. LGIV: *ced-2(e1752), ced-10(n1993rf), ced-5(n1812), him-8(e1489), ced-3(n2427rf), ced-3(n717lf), ced-3(n3692del)*. LGV: *him-5(e1490), him-5(e1467ts), egl-1(n1084 n3082), unc-76(e911)*. LGX: *nIs349* [P_{ceh-28}*::mCherry, lin-15AB(+)*]. Extrachromosomal arrays: *nEx2344* [P_{evl-20}*::mCherry::PH, unc-76(+)*] (this study). *ts*, temperature-sensitive; *gf*, gain-of-function; *rf*, reduction-of-function; *lf*, total loss-of-function; and *del*, deletion.

General microscopy.

To identify B.alapaav and B.arapaav, we traced their cell lineages in several animals to determine their distinctive positions as previously described (Sulston and Horvitz 1977). Once it was ascertained that *nIs343* was expressed in the dying B.al/rapaav in all genetic backgrounds studied, we used it as a marker to score the survival of B.al/rapaav in mutant animals. We mounted animals in 20-60 mM sodium azide on 4% agar pads. We estimated the age of animals based on the following criteria: early fourth larval stage animals had exited the L3/L4 lethargus but had no developed hook structure (approximately 34-37 hours after hatching); mid-fourth larval stage animals had a developed hook structure, and the tail was starting to retract

(approximately 37-40 hours after hatching); late fourth larval stage animals had visible ray tips or more developed rays, but had not entered the period of lethargus between the fourth-larval and adult stages (approximately 40-43 hours after hatching). We first used Nomarski differential interference contrast optics to putatively identify B.al/rapaav and categorize its morphology and then confirmed B.al/rapaav identity by checking for *nIs343* expression using a fluorescence-equipped Axioskop II compound microscope (Zeiss, Oberkochen, Germany). Images were acquired with an ORCA-ER CCD camera (Hamamatsu, Hamamatsu City, Japan) using OpenLab software (Agilent, Lexington, MA).

To score engulfment, $P_{evl\text{-}20}$*::mCherry::PH* animals were anesthetized with 60 mM sodium azide and mounted on 4% agar pads. For data described in Figure 7 and Figure 8A, animals were imaged at 0.2-0.25 μm intervals in the region containing B.alapaav and B.arapaav using a Zeiss LSM 510 confocal microscope. For data described in Figure 8D, P12.pa boundaries were visualized using an Axioskop II compound microscope (Zeiss). A cell was considered to be engulfed if it looked to be within another cell's boundaries, as visualized by mCherry::PH. Engulfing cell identity was deduced by comparison of nuclear and cell boundary positions to those in the dataset "JSG_male_tail" in the WormImage Database on WormAtlas (Altun *et al.*).

Image processing was done using Fiji.

Single-molecule fluorescence *in situ* hybridization.

Fixation of larval animals, conjugation of fluorescent probes to and purification of oligo probes, hybridization and imaging were performed as previously described (Denning *et al.* 2013). The *egl-1* set of probes included 21 20-nucleotide probes complementary to regions in the second and third exons and 3' untranslated region of *egl-1.* The *egl-1* probe set was conjugated to the fluorophore Alexa 594 (Invitrogen, Carlsbad, CA). The *ced-3* set of probes included 48 20-nucleotide probes complementary to regions in all exons of *ced-3*. The *ced-3* probe set was conjugated to Alexa 594 for images and analysis in Figure 2E, and Figure 4 and to Cy5 (Invitrogen) for Figure 2C,D and Figure 3. The *ced-4* set of probes included 48 20-nucleotide probes complementary to all exons of the short pro-apoptotic isoform of *ced-4* conjugated to Alexa 594. The *ced-9* set of probes included 48 20-nucleotide probes complementary to all exons and 3' untranslated region of *ced-9* conjugated to Cy5. Image processing was done using Fiji.

B.alapaav and B.arapaav were identified based on the positions of DAPI-stained nuclei in animals estimated to be between 33 and 37 hours of age based on the progress of the ray lineage cell divisions and deaths. Transcripts within the B.alapaav and B.arapaav nuclei were manually identified and quantified. We analyzed nuclear transcripts because we could not unambiguously determine to which cell cytoplasmic transcripts belonged.

Laser ablation.

First and second larval stage animals were anesthetized with 20 mM sodium azide and mounted on 4% agar pads. P12.pa was identified by Nomarski differential interference contrast optics and killed using the laser system described by Avery and Horvitz (1987). Mock-ablated animals were mounted along with ablated animals, but the laser was aimed next to the animal. The next day, recovered animals that had reached the fourth larval stage were remounted with food on 4% agar pads in 10% polyvinyl pyrrolidone in M9 buffer and checked for normal developmental rate, the absence of P12.pa and the lack of other visible damage before scoring. Slides were sealed with petroleum jelly to prevent drying. Animals were maintained at 20°C and observed until the B.al/rapaav death or until the tips of the rays were visible (late fourth larval stage), at which point they were scored as the B.al/rapaav death failing to occur.

Plasmid construction.

To create the $P_{evl\text{-}20}$*::mCherry::PH* transgene, a 3.1 kb fragment 5' of *evl-20* was PCR amplified using primers with *Pst*I and *Nhe*I restriction sites incorporated on the 5' ends to facilitate cloning. The purified PCR amplicon was digested with *Nhe*I and *Pst*I, the plasmid pDD111 was digested with *Xba*I and *Pst*I, and the purified digestion products were ligated together (pDD111 is a plasmid containing $P_{egl\text{-}1}$*::mCherry::PH::unc-54 3' UTR* that was a gift from Dan Denning). An out-of-frame start codon was inadvertently introduced, which was removed by site-directed

mutagenesis. The plasmid was injected at 10 ng/µL into *him-5(e1467ts) unc-76(e911)* animals with 50 ng/µL of *unc-76(+)* as a co-injection marker.

Statistical notes.

Target sample sizes were selected prior to evaluating significance. Sample sizes varied slightly depending on the number of animals available of the appropriate age for scoring. Data for each experiment were collected over multiple days and pooled. For smFISH experiments, animals were excluded from analysis if the signal was weak in other tissues (*e.g.* germline for *ced-3* and Rn.aap cells for *egl-1*). To calculate *P*-values we used the two-tailed Student's *t*-test for quantitative data (*i.e.* smFISH transcript counts and fluorescence intensity data) and the two-tailed Fisher's test to compare proportions (*i.e.* fraction of worms with B.al/rapaav survival, GFP fluorescence or a given morphological appearance). Reported *P*-values are corrected for multiple hypothesis testing by the Bonferonni correction. When unspecified, reported *P*-values were calculated by comparison with the wild-type strain containing the same *him* mutation.

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Figure 1. B.alapaav and B.arapaav are left-right homologs and the B.al/rapaav death is dependent on engulfment and suicide genes. (**A**) The B.alapaav and B.arapaav cell lineages. Figure adapted from Sulston *et al.*, 1980. (**B**) B.alapaav and B.arapaav are located close to each other and to the engulfing cell P12.pa in the developing male tail. Cell nuclei were traced from a DIC image of an otherwise wild-type male of genotype *nIs343[*$P_{egl\text{-}1}$*::4xNLS::gfp]; him-8* just before the fourth larval stage, about 34 hours after hatching. Scale bar: 10 μm. (**C**) A schematic of the movements of B.alapaav and B.arapaav (blue) as viewed from above the animal. At the time of their generation, B.alapaav and B.arapaav are located to the left and right sides of the rectum. They move closer to the midline, ventral to the rectum. Eventually, the B.al/rapaav homolog will move closer to the midline, and B.al/rapaav typically moves slightly posterior and further from the midline. The B.al/rapaav homolog survives, and B.al/rapaav will undergo programmed cell death. In this diagram, B.alapaav is the dying B.al/rapaav and B.arapaav is the surviving B.al/rapaav homolog. (**D**) The percentages of late fourth larval stage males with a living $P_{egl\text{-}1}$*::4xNLS::gfp*-expressing secondary B.al/rapaav. All genotypes include *nIs343* and some also contain †: *him-5(e1490),* ‡: *him-8,* °: *nIs349,* §: *lon-1(e1820) dpy-17,* ❖: *lon-1(e185),* ★: *unc-32*. n.s.: $P > 0.5$, *: $P < 0.005$, **: $P < 10^{-5}$.

Figure 1

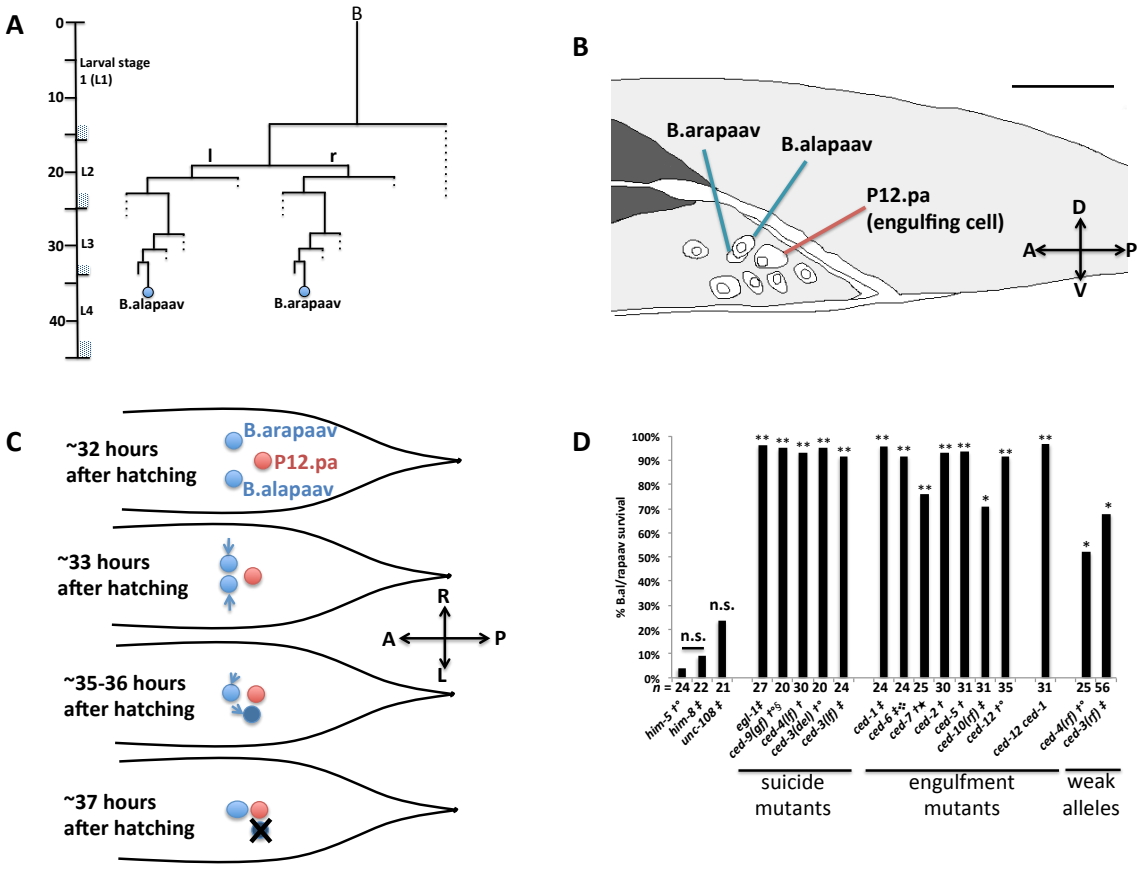
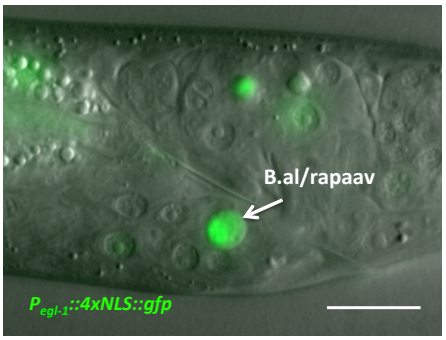

A
0
Larval stage
1 (L1)
10
20
L2
30
L3
40
L4
B
l
r
B.alapaav
B.arapaav
B
B.arapaav
B.alapaav
P12.pa
(engulfing cell)
D
A
P
V
C
~32 hours
after hatching
B.arapaav
P12.pa
B.alapaav
~33 hours
after hatching
R
A
P
L
~35-36 hours
after hatching
~37 hours
after hatching
D
% B.al/rapaav survival
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
n.s.
n.s.
n = 24 22 21 27 20 30 20 24 24 24 25 30 31 31 35 31 25 56
him-5 †*
him-8 ‡
unc-108 ‡
egl-1‡
ced-9(gf) †*§
ced-4(lf) †
ced-3(de) †*
ced-3(lf) ‡
ced-1 ‡
ced-6 ‡*§
ced-7 †*
ced-2 †
ced-5 †
ced-10(rf) ‡
ced-12 †*
ced-12 ced-1
ced-4(rf) †*
ced-3(rf) ‡
suicide
mutants
engulfment
mutants
weak
alleles

Figure 2. Cell-death genes are expressed in B.al/rapaav. (**A**) GFP under the control of the *egl-1* promoter is expressed brightly in B.al/rapaav in the tail of an early fourth larval stage "wild-type" male of genotype *nIs343; him-8*. Scale bar: 10 μm. (**B**) The average intensity of P_{egl-1}::4xNLS::GFP in the nucleus is higher in the dying B.al/rapaav cell than in the surviving B.al/rapaav homolog in early fourth larval stage animals. The B.al/rapaav fate was assigned based on partial cytoplasmic refractility and/or nuclear position and distance from the midline of the animal. Average fluorescent intensity within the nucleus was measured for confocal images of B.alapaav, B.arapaav and P12.pa in each animal, and the average background intensity was subtracted from each. A.U., arbitrary units. All genotypes include *nIs343,* and some also contain †: *him-5(e1490),* ‡: *him-8,* °: *nIs349.* (**C-E**) Proapoptotic genes *egl-1* and *ced-3* are not highly expressed in the surviving B.al/rapaav homolog (**C**) but are highly expressed in the dying B.al/rapaav (**D**)**.** These two images were taken from the same animal, approximately 1.6 μm apart. Anterior, left; dorsal, top. Scale bars: 10 μm. These animals were of the genotype *nIs343; him-5(e1490); nIs349.* (**E**) Quantification of nuclear mRNA transcripts of *egl-1* and *ced-3* in B.al/rapaav and the B.al/rapaav homolog. Only nuclear transcripts were counted, because we could not unambiguously determine to which cell non-nuclear transcripts belonged. Condensed chromatin (as visualized by DAPI, which stains DNA and shows the chromatin in a dying cell to be slightly brighter and smaller in volume than that in a living cell), a position to the left or right of P12.pa, or expression of P_{egl-1}*::4xNLS::gfp* was each interpreted as indicating the secondary

cell fate. All animals were of the genotype *nIs343*, *him-5(e1490), nIs349.* *: $P < 0.05$, **: $P < 5\times10^{5}$.

Figure 2

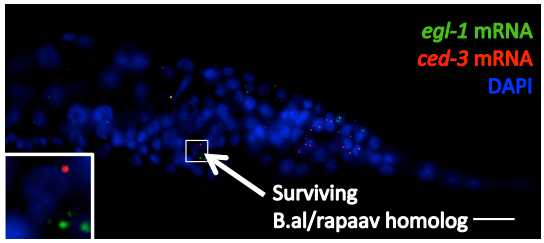
A



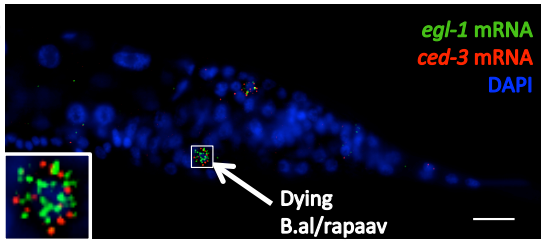
B

	Average nuclear P_{egl-1}::4xNLS::GFP intensity values (A.U.) ± SD				
	P12.pa	B.al/rapaav homolog	B.al/rapaav	*n*	*P*-value
him-5 †°	1.1 ± 1.0	2.3 ± 2.3	26.7 ± 18.6	26	2.2×10^{-6}
him-8 ‡	0.4 ± 0.8	0.5 ± 0.9	16.9 ± 12.4	25	2.2×10^{-6}

C



D



E

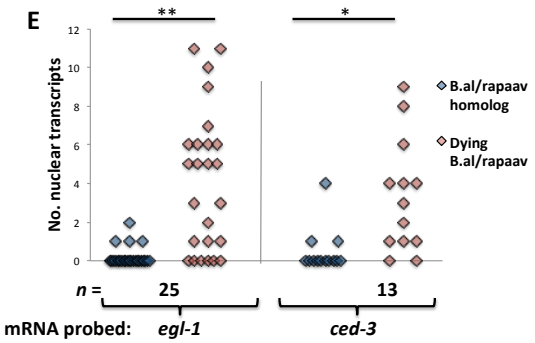


Figure 3 Cell death genes *ced-9* **(A)** and *ced-4* **(B)** are expressed broadly, including in the male tail and the germline. Animals are of the genotype *nIs343; him-8.* Magenta: *ced-9::*Cy5. Yellow: *ced-4::*Alexa594. Blue: DAPI. Scale bar: 10 μm.

Figure 3

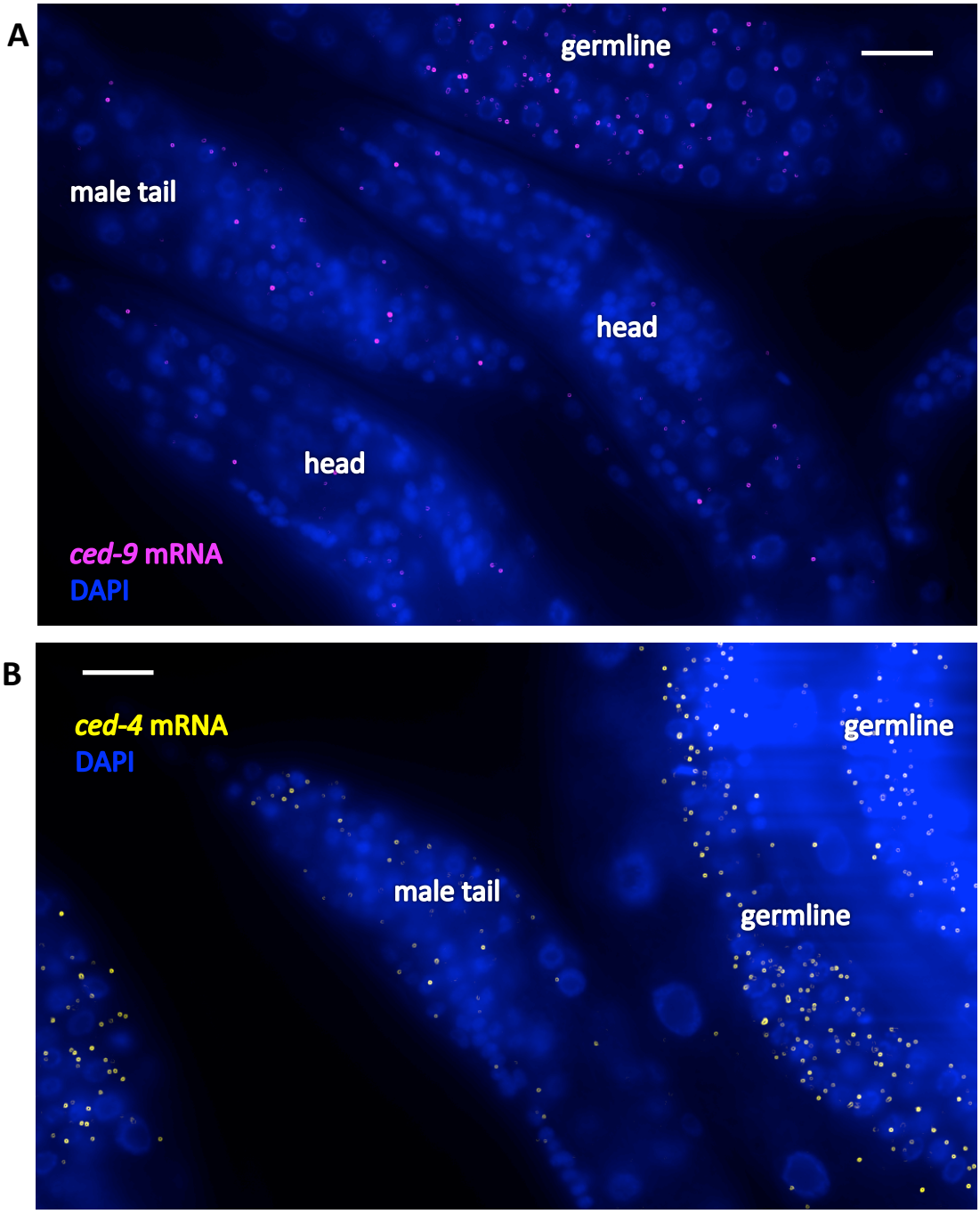
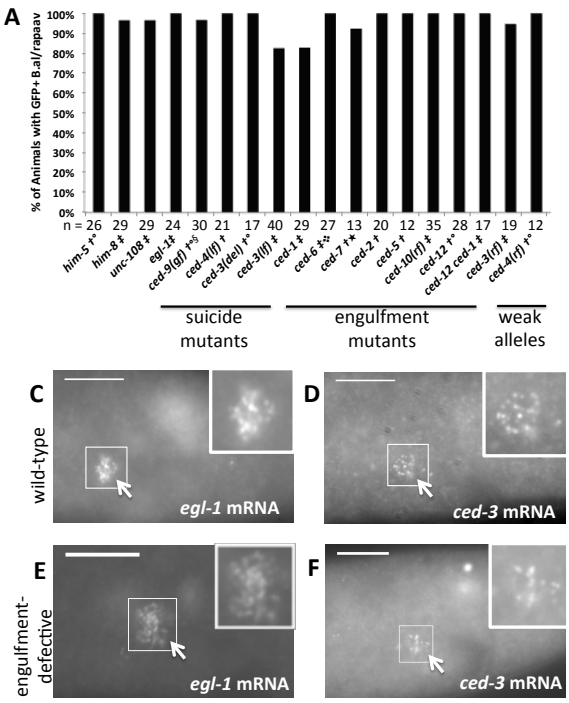

A
germline
male tail
head
head
ced-9 mRNA
DAPI
B
ced-4 mRNA
DAPI
germline
male tail
germline

Figure 4. B.al/rapaav and the B.al/rapaav homolog express pro-apoptotic genes differentially in cell-death mutants. All genotypes include *nIs343,* and some also contain †: *him-5(e1490),* ‡: *him-8,* °: *nIs349,* §: *lon-1(e1820) dpy-17,* ❖: *lon-1(e185)*, ★: *unc-32.* (**A**) B.al/rapaav expresses $P_{egl\text{-}1}$*::4xNLS::gfp* in all genetic backgrounds studied. % animals with $P_{egl\text{-}1}$*::4xNLS::gfp* expression in death-fated B.al/rapaav during the early fourth larval stage, before the B.al/rapaav death occurs. (**B**) The average intensity of $P_{egl\text{-}1}$::4xNLS::GFP in the nucleus is higher in B.al/rapaav than in the B.al/rapaav homolog in early fourth larval stage animals defective for cell death genes. A.U., arbitrary units. (**C-F**) *egl-1* (**C,E**) and *ced-3* (**D,F**) transcripts are detectable in the presumptive B.al/rapaav (white arrow) in the tails of early fourth larval stage wild-type (**C,D**) and engulfment-defective (**E,F**) males. The genotype in (**C**) was *nIs343; him-8*; (**D**), *nIs343; him-5(e1490); nIs349*; (**E,F**), *ced-12 ced-1; nIs343; him-8.* Scale bars: 10 μm. (**G**)*, ced-3* and *egl-1* are differentially expressed between B.alapaav and B.arapaav. Only nuclear transcripts were counted. For each animal, the cell with the larger number of transcripts was classified as "high expression B.al/rapaav" and the other cell was classified as "low expression B.al/rapaav homolog." When both cells had the same number of transcripts, one was arbitrarily labeled high and one was labeled low. This panel includes data from Figure 2E. Animals contained *nIs343* and †:*him-5(e1490),* ‡:*him-8,* °:*nIs349*. *: $P < 0.001$.

Figure 4



B

	Average nuclear P_{egl-1}::4xNLS::GFP intensity values (A.U.) ± SD				
	P12.pa	B.al/rapaav homolog	B.al/rapaav	*n*	*P*-value
ced-12 ced-1 ‡	0.4 ± 0.5	2.9 ± 3.2	25.7 ± 19.5	25	4.7×10^{-6}
ced-3(lf) ‡	0.2 ± 0.3	3.0 ± 3.9	39.9 ± 30.9	25	5.8×10^{-6}
ced-3(del) †°	0.1 ± 0.3	2.1 ± 3.2	32.3 ± 23.9	26	1.1×10^{-6}

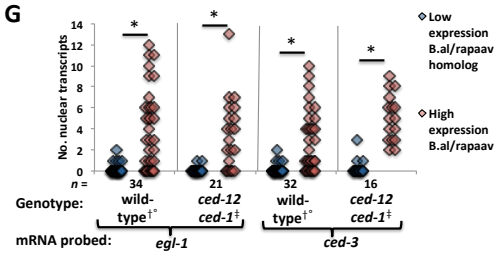


Figure 5. The surviving B.al/rapaav is morphologically different in engulfment-gene and suicide-gene mutants. (**A-C**), The morphology of a dying B.alapaav changes over time; *him-8* fourth larval stage male. Arrows and insets show B.alapaav. Scale bars: 10 μm. (**A**) 1 hour after its generation B.alapaav was non-refractile. (**B**) 3.5 hours after its generation B.alapaav was rounded and the cytoplasm was refractile, but the nucleus was non-refractile. (**C**) 4 hours after its generation B.alapaav was a refractile corpse. (**D-F**) % animals in which B.al/rapaav displayed the morphology seen in images (**C**), (**B**) and (**A**), respectively, in mid-fourth larval stage animals. Animals with no visible secondary B.al/rapaav, presumably because this cell had already been degraded, were excluded from this analysis. All genotypes include *nIs343* and some also contain †: *him-5(e1490),* ‡: *him-8,* °: *nIs349,* ❖: *lon-1(e185),* ★: *unc-32,* §: *lon-1(e1820) dpy-17*. (**D**) n.s.: $P > 0.5$, *: $P < 0.005$. (**D**) Wild-type vs. engulfment mutants (*ced-1, ced-6, ced-7, ced-2, ced-5, ced-12* and *ced-1 ced-12*): $P < 10^{-17}$, wild-type vs. suicide mutants (*egl-1, ced-9(gf), ced-4(lf), ced-3(del)* and *ced-3(lf)*): $P < 10^{-15}$, engulfment vs. suicide mutants: $P > 0.5$. (**E**) Wild-type vs. engulfment mutants: $P < 10^{-11}$, wild-type vs. suicide mutants: $P > 0.5$, engulfment vs. suicide mutants: $P < 10^{-31}$. (**F**) Wild-type vs. engulfment mutants: $P > 0.5$, wild-type vs. suicide mutants: $P < 10^{-12}$, engulfment vs. suicide mutants: $P < 10^{-30}$. ***P*-**values between classes of mutants are by two-tailed Fisher's exact test using data pooled within genotypic classes (wild-type, engulfment mutants and suicide mutants). (**G-H**), Representative images of the morphology of the undead B.al/rapaav in *ced-12 ced-1* engulfment (**G**) and *ced-3(lf)* suicide (**H**) mutant backgrounds.

Figure 5

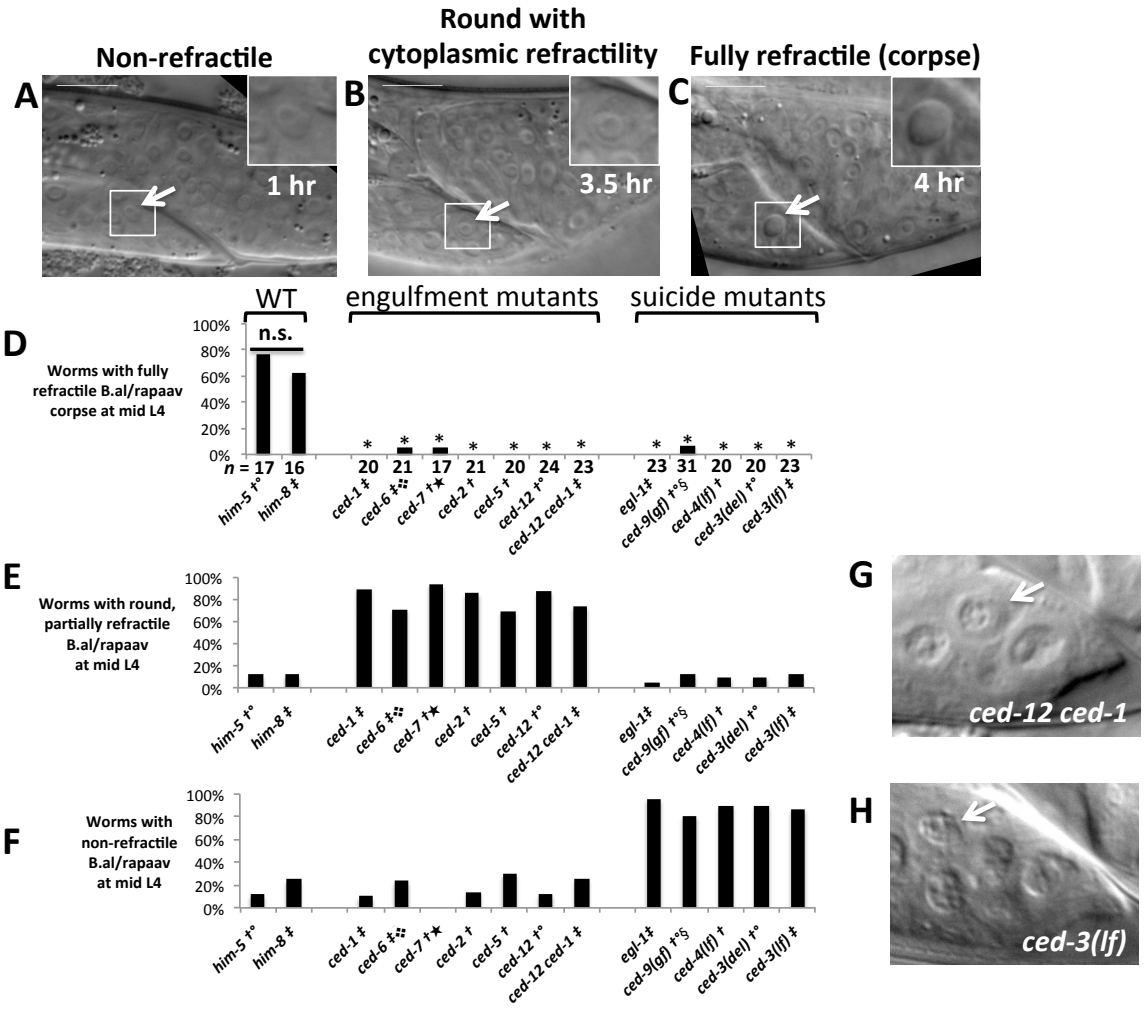

Non-refractile
Round with cytoplasmic refractility
Fully refractile (corpse)
A
B
C
1 hr
3.5 hr
4 hr
WT
engulfment mutants
suicide mutants
D
Worms with fully refractile B.al/rapaav corpse at mid L4
n.s.
n = 17 16 20 21 17 21 20 24 23 23 31 20 20 23
him-5 †° him-8 ‡ ced-1 ‡ ced-6 ‡⁘ ced-7 †★ ced-2 † ced-5 † ced-12 †° ced-12 ced-1 ‡ egl-1‡ ced-9(gf) †°§ ced-4(lf) † ced-3(del) †° ced-3(lf) ‡
E
Worms with round, partially refractile B.al/rapaav at mid L4
F
Worms with non-refractile B.al/rapaav at mid L4
G
ced-12 ced-1
H
ced-3(lf)

Figure 6. The undead B.al/rapaav cell in engulfment mutants appears more similar to a living B.al/rapaav than to a B.al/rapaav corpse. (**A-C**) A B.al/rapaav cell in the early fourth larval stage before cell death is complete lacks refractility in the nucleus (blue arrowheads) (**A**), has P_{egl-1}::4xNLS::GFP in the nucleus (blue arrowheads) but not in the rest of the cell (white arrows) (**B**), and has P_{evl-20}::mCherry::PH localized to the membrane (**C**). (**D-F**) A B.al/rapaav cell corpse in the mid-fourth larval stage has refractility throughout the cell (white arrows) (**D**), P_{egl-1}::4xNLS::GFP throughout the cell (**E**), and P_{evl-20}::mCherry::PH throughout the cell (**F**). (**G-I**) An undead B.al/rapaav cell in the late fourth larval stage of an engulfment mutant lacks refractility in the nucleus (blue arrowheads) (**G**), has P_{egl-1}::4xNLS::GFP in the nucleus (blue arrowheads) but not the rest of the cell (white arrows) (**H**), and has P_{evl-20}::mCherry::PH localized to the membrane (**I**). Animals were of genotypes *nIs343; him-5(e1467ts) unc-76; nIs349; nEx2344* (**A-F**) or *nIs343; ced-10 him-8; nIs735* (**G-I**). Scale bars: 10 μm. (**J**) A B.al/rapaav cell in the early fourth larval stage has diffuse chromatin similar to neighboring living cells. (**K**) A dying or dead B.al/rapaav cell in the mid-fourth larval stage has condensed chromatin. (**L**) An undead B.al/rapaav cell in the late fourth larval stage of an engulfment mutant has diffuse chromatin similar to neighboring living cells. Scale bars: 10 μm. Animals were of the genotypes *nIs343; him-5(e1490); nIs349* (**J,K**) or *ced-12 ced-1; nIs343; him-8* (**L**).

Figure 6

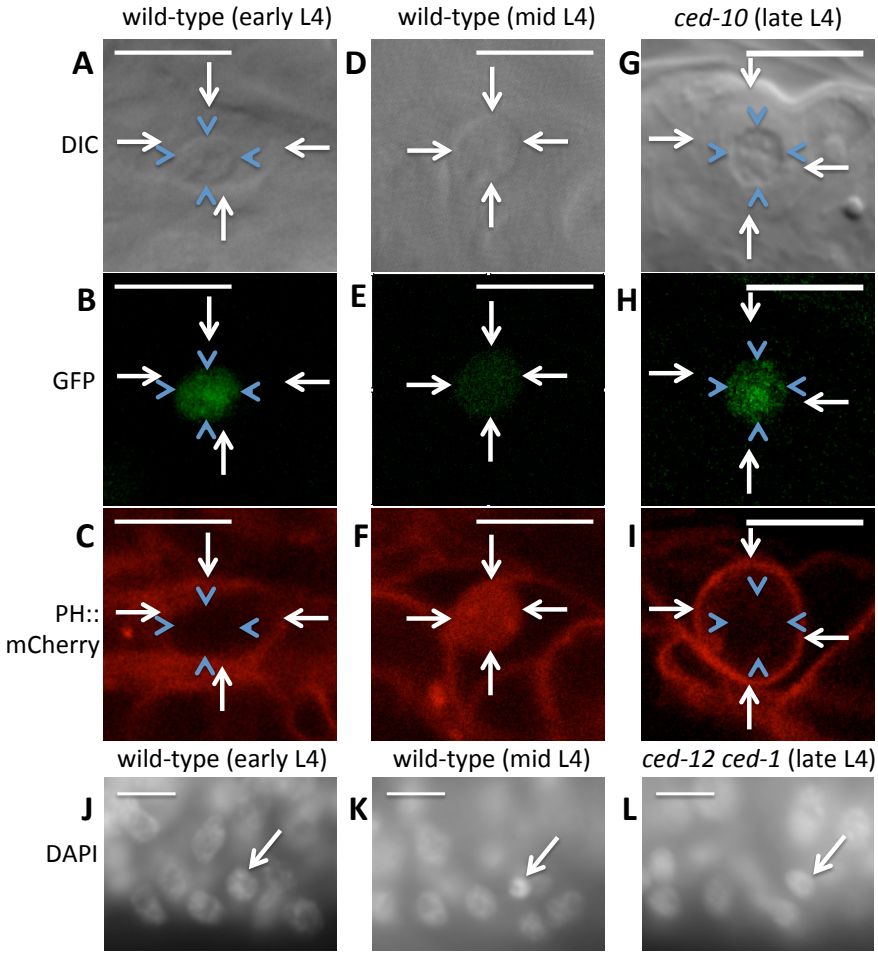

wild-type (early L4)
wild-type (mid L4)
ced-10 (late L4)
A
D
G
DIC
B
E
H
GFP
C
F
I
PH::
mCherry
wild-type (early L4)
wild-type (mid L4)
ced-12 ced-1 (late L4)
J
K
L
DAPI

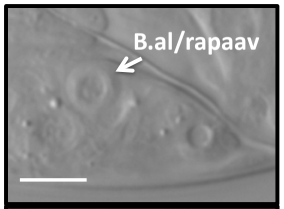
Figure 7. P12.pa ablation does not prevent initiation of the B.al/rapaav death. (**A**) Percentages of P12.pa-ablated and mock-ablated males that have cytoplasmic refractility in B.al/rapaav; $P_{egl\text{-}1}$*::4xNLS::gfp* expression in B.al/rapaav; or death of B.al/rapaav. Percentage of survival of B.al/rapaav in mock-ablated animals is not significantly different from that of untreated animals in Figure 1D (P = 0.3). (**B,C**) A P12.pa-deficient animal in which the secondary B.al/rapaav (arrow) had cytoplasmic refractility (**B**) and had $P_{egl\text{-}1}$*::4xNLS::gfp* expression (**C**), indicating that the cell-death process was initiated. Scale bars: 10 μm. All animals in panels (**A-C**) were of genotype *nIs343; him-8*. **(D),** A round, undead B.al/rapaav that was not engulfed in a P12.pa-deficient animal. White arrow points to the undead B.al/rapaav; the only membrane (red) visible around the B.al/rapaav nucleus (green) is that of the undead cell (since only one nucleus is located within that membrane), and no other cell's membrane appears to enclose it in any plane. **(E)**, A B.arapaav corpse that was engulfed by B.alapaav in a P12.pa-deficient animal. The B.arapaav corpse (white arrow) appears to be completely inside the boundaries of the B.alapaav membrane (black in right panel). This membrane can be assigned to B.alapaav, because it contains the B.alapaav nucleus (black arrow). Scale bars: 10 μm. **(F)** Outcomes of the secondary B.al/rapaav in P12.pa-ablated animals, showing that all surviving cells were unengulfed while most dying cells were engulfed. All animals in panels **(D-F)** were of the genotype *nIs343; him-5(e1467ts) unc-76; nIs349; nEx2344*.

Figure 7

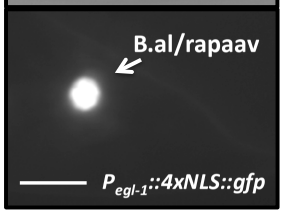
A

	P12.pa-ablated animals (*n*)	Mock-ablated animals (*n*)
Cytoplasmically refractile B.al/rapaav	88.5% (26)	100% (9)
P_{egl-1}*::gfp* expressed in B.al/rapaav	100% (27)	100% (9)
B.al/rapaav death	39% (23)	75% (8)

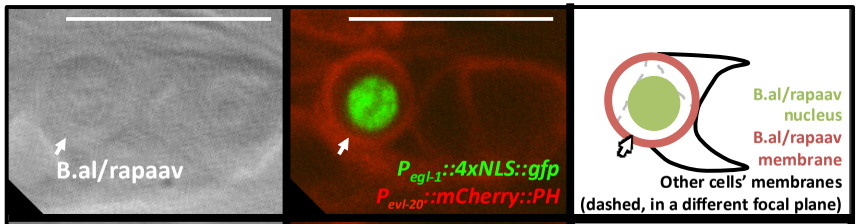
B



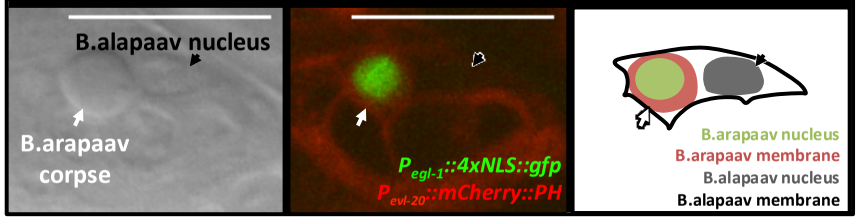
C



D



E



F

Outcomes of B.al/rapaav in P12.pa-ablated animals (*n* = 22)	Status of B.al/rapaav Engulfment	
	Unengulfed	Engulfed
Death	9%	64%
Survival	27%	0%

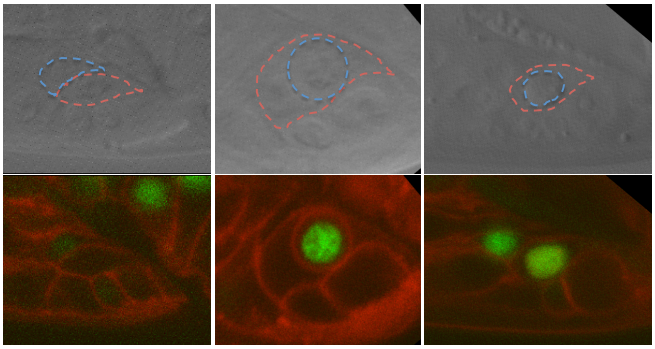
Figure 8. Engulfment precedes the B.al/rapaav death. (**A**) All non-refractile B.al/rapaav cells are unengulfed, most cells that are round with partial cytoplasmic refractility are engulfed by P12.pa and all fully refractile cells are engulfed by P12.pa. Representative DIC and fluorescent images corresponding to each of the B.al/rapaav morphology classes are shown, with the P12.pa (dashed red line) and B.al/rapaav cell membranes (dashed blue line) outlined (based on $P_{evl\text{-}20}$::mCherry::PH signal). (**B,C**) A B.arapaav cell with partial cytoplasmic refractility (white arrow) visualized by (**B**) DIC and (**C**) electron microscopy (blue arrowheads). (**C**) The B.arapaav cell is engulfed by P12.pa (red arrows) but otherwise does not display obvious signs of cell death. The animal was of genotype *him-8.* **(D)** B.al/rapaav does not die in a weak engulfment mutant without being engulfed. Engulfment and cell-killing are not independent events ($P = 0.0075$, Fisher's exact test). B.al/rapaav was classified as being dead or undead based on morphology as seen with DIC optics and was then examined to determine if it were inside of P12.pa based on $P_{evl\text{-}20}$::mCherry::PH. Animals were of the genotype *nIs343; ced-10 him-8; nIs735*.

Figure 8

A

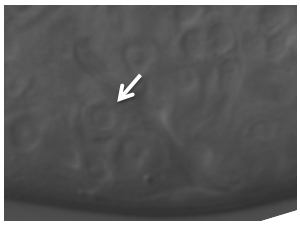
	B.al/rapaav morphology		
	Non-refractile	Round with cytoplasmic refractility	Fully refractile
	n = 10	*n* = 16	*n* = 19
Unengulfed	100%	12.5%	0%
Engulfed by P12.pa	0%	87.5%	100%
Engulfed by other cell	0%	0%	0%

DIC image
B.al/rapaav membrane
P12.pa membrane

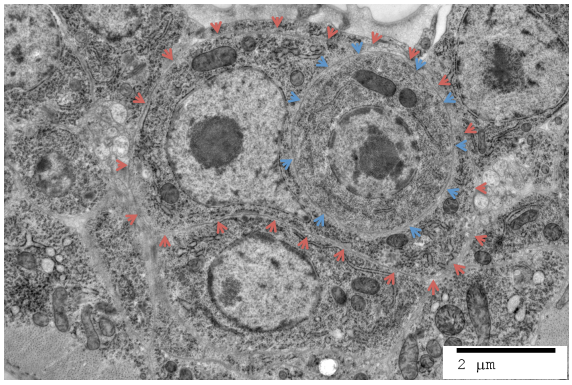


P*$_{egl-1}$*::4xNLS::gfp
P*$_{evl-20}$*::mCherry::PH

B



C



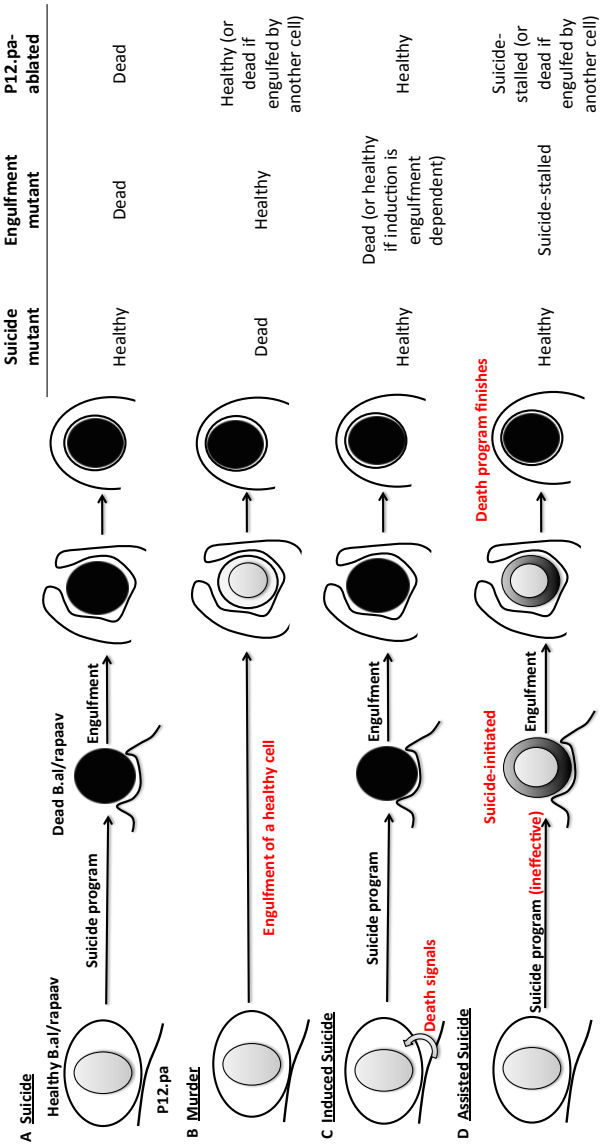
D

Outcomes of B.al/rapaav in *ced-10(n1993rf)* animals	**Status of B.al/rapaav engulfment**	
	Unengulfed	**Engulfed**
Corpse (*n* = 13)	0%	100%
Undead (*n* = 10)	50%	50%

Figure 9. Models of alternative modes of B.al/rapaav cell death. **(A)** Suicide: B.al/rapaav initiates the suicide program and undergoes programmed cell death, resulting in a fully-refractile corpse, which is engulfed by the neighboring cell P12.pa. A suicide cell death would occur in engulfment mutants and in P12.pa-ablated animals but not in suicide-pathway mutants. Our data are inconsistent with this model, as the death does not occur in engulfment mutants. **(B)** Murder: B.al/rapaav is healthy until it is engulfed by P12.pa, after which it dies. This form of cell death would occur in suicide-pathway mutants but not in engulfment mutants or P12.pa-ablated animals (unless engulfed and killed by a different neighboring cell). Our data are inconsistent with this model, as the death does not occur in suicide mutants. **(C)** Induced suicide: B.al/rapaav is signaled by P12.pa to induce the suicide program. This form of cell death would not occur in suicide-pathway mutants or P12.pa-ablated animals but would occur in engulfment mutants, (unless the death-inducing signal requires engulfment genes, which is not the case for B.al/rapaav, since the cell-death suicide genes are expressed in engulfment mutants). Our data are inconsistent with this model, as initiation of the B.al/rapaav death process is not dependent on the presence of P12.pa or the activities of the engulfment genes (the cell-death suicide genes are expressed in both cases, and death-related morphological changes can be seen in B.al/rapaav in engulfment mutants and in most animals in which P12.pa was ablated). **(D)** Assisted suicide: B.al/rapaav initiates the suicide program, which is ineffective at causing programmed cell death and results in a suicide-stalled cell. Once B.al/rapaav is engulfed, the death reaches completion. This form of cell death would not occur in

suicide mutants, engulfment mutants or P12.pa-ablated animals (unless engulfed by another cell). The suicide-stalled cell in an engulfment mutant might show signs of cell-death initiation. Our data are consistent with this model.

Figure 9


Predicted phenotypes of B.al/rapaav
Suicide mutant
Engulfment mutant
P12.pa-ablated
A Suicide
Healthy B.al/rapaav
Suicide program
Dead B.al/rapaav
Engulfment
P12.pa
Healthy
Dead
Dead
B Murder
Engulfment of a healthy cell
Dead
Healthy
Healthy (or dead if engulfed by another cell)
C Induced Suicide
Suicide program
Death signals
Engulfment
Healthy
Dead (or healthy if induction is engulfment dependent)
Healthy
D Assisted Suicide
Suicide program (ineffective)
Suicide-initiated
Engulfment
Death program finishes
Healthy
Suicide-stalled
Suicide-stalled (or dead if engulfed by another cell)

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Chapter 3

An RNAi Screen for Essential Genes with a Role in Programmed Cell Death

Summary

Although the genetic control of programmed cell death in *C. elegans* has been extensively studied, there have been no systematic attempts to identify essential genes with a role in programmed cell death. Such genes are not easily identified by a standard genetic screen. I performed an RNAi screen to inhibit essential genes and determine if any treatments prevented the death of the Rn.aap cells in the male tail. From this screen I identified many candidate cell-death genes, but I was unable to identify a global role in cell death for any of these genes.

Introduction

Programmed cell death occurs during the normal development of many organisms. The *C. elegans* cell-death pathway has been extensively studied for a variety of cells that are fated to die. During programmed cell death, caspases are activated in the dying cell. The cell corpse is then engulfed by a neighboring cell and degraded.

Previous screens for genes involved in cell death have not been designed to systematically identify essential genes with a role in cell death, as loss or reduced function of essential genes causes lethality, decreased viability, and/or slow growth. In a standard, non-clonal screen of the second generation after mutagenesis, such mutants will be low in number; outcompeted by other, healthier animals; or will not survive to the age suitable for scoring a mutant phenotype. The essential cell death gene *ced-9* was first identified by a gain-of-function mutation, which did not cause

lethality (Hengartner *et al.*, 1992). It is unknown if there exist more essential genes with roles in programmed cell death.

Most somatic cell deaths in *C. elegans* occur during embryonic development or in the early larval stages, with the exception of several male-specific cell deaths, including 18 Rn.aap deaths in the ray lineage, which occur during the fourth larval stage (Sulston and Horvitz, 1977; Sulston *et al.*, 1980, 1983). These late cell deaths provide an approach to examine essential genes for a role in programmed cell death, as RNA interference (RNAi) treatment after hatching could eliminate gene function before the death would occur but after embryogenesis has been successfully completed, allowing the animals to develop. I performed an RNAi screen to knock down the function of 1,132 essential genes and examine their role in the Rn.aap deaths. The design and outcomes of this RNAi screen are described below.

Results

Optimizing the screen

The nine bilateral pairs of rays in the male tail are generated through a stereotyped pattern of cell divisions from nine precursor cells on the left and right (R1-R9, or Rn) (Sulston and Horvitz, 1977), see Figure 1. Each Rn cell produces two neurons, a structural cell, a hypodermal cell and Rn.aap, a cell that dies (Sulston *et al.*, 1980). The Rn.aaa cells differentiate into A-type neurons, six of which are dopaminergic: the left and right R5.aaa, R7.aaa and R9.aaa (Sulston *et al.*, 1980). To

determine if undead Rn.aap cells in a cell death-defective animal might adopt the identity the Rn.aaa sister cells, I examined green fluorescent protein (GFP) reporters known to be expressed in dopaminergic neurons (Koo *et al.*, 2011; Lints and Emmons, 1999)

I examined two pre-existing markers associated with dopaminergic fate, *nIs143[*$P_{dat\text{-}1}$*::dat-1::gfp]* and *nIs116[*$P_{cat\text{-}2}$*::gfp]* in backgrounds normal and defective for cell death. *him-8; nIs143* animals had an average of 5.5 ± 0.8 GFP-positive cells in the tail (n = 33) while *him-8 ced-3; nIs143* animals had an average of 7.6 ± 1.5 GFP-positive cells (n = 39), a significant increase ($p = 10^{-10}$). Similarly, *him-8; nIs116* animals had an average of 6.0 ± 0.0 GFP-positive cells in the tail (n = 6) while *him-8 ced-3; nIs116* animals had an average of 8.0 ± 1.4 GFP-positive cells (n = 5), also a significant increase (p = 0.03). These data are consistent with the undead Rn.aap cells sometimes adopting a dopaminergic fate.

The GFP from *nIs143* and *nIs116* was dim and formed aggregates, respectively, which made them difficult to score accurately (data not shown). To create a marker for the undead Rn.aap cells that could be more easily scored, I created a transcriptional reporter using the same promoter region used to create *nIs143* fused to the sequence for nuclearly-localized GFP. I integrated this transgene to create *nIs583*, which maps to Chromosome III, and *nIs584*, which maps to Chromosome I. The $P_{dat\text{-}1}$*::4xNLS::gfp* reporter gave a strong, clear signal in 6.0±0.2 cells in *nIs583; him-8* animal tails (range = 5-7, n = 103) and 10.8±1.2 cells in *nIs343; him-8 ced-3* mutant tails (range = 6-13, n = 95), consistent with it being expressed in R5.aaa, R7.aaa, R9.aaa, and their undead sisters, on the left and right sides of the tail

(Figure 2).

RNAi treatment has a weaker effect in neurons, but there are various mutations that cause increased sensitivity to RNAi (Lehner *et al.*, 2006; Schmitz *et al.*, 2007; Simmer *et al.*, 2002; Wang *et al.*, 2005). Since the Rn.aap cells are in a sublineage that creates neurons, I tested some of these RNAi hypersensitive mutations to determine the most sensitive strain for RNAi knockdown in Rn.aap cells. I created strains containing *nIs583* or *nIs584* together with *hpl-2, lin-35, rrf-3* or *nre-1* and *lin-15b* and performed RNAi against known cell-death genes *ced-3*, *ced-4*, and *egl-1* to determine which background gave the strongest Ced phenotype (Figure 3). The strain containing *rrf-3* had silencing of the *nIs583* transgene and was excluded from further analysis. I found that *lin-35* animals had the strongest Ced phenotype after RNAi targeting *ced-3*, *ced-4,* or *egl-1*, while maintaining a wild-type phenotype after feeding with an empty RNAi vector as a negative control. In addition to increasing RNAi sensitivity *lin-35* mutation reduces somatic cell death (in a sensitized background) and germ cell death (Reddien *et al.*, 2007; Schertel and Conradt 2007); its role in cell death could at least in part contribute to the enhanced Ced phenotype after RNAi targeting cell death genes, but its role is too minor to cause cell survival of Rn.aap cells on its own or after feeding with the empty RNAi vector. *lin-35* animals also had robust knockdown of GFP in the Rn.aaa cells after RNAi treatment targeting the GFP gene (79% of animals had four or fewer cells with visible GFP, n = 33). I also found that the RNAi was stronger with standard IPTG concentration (1 mM) than with a higher IPTG concentration (2.5 mM) (Figure 4).

Screening for essential genes involved in cell death

I used the *lin-35; nIs583; him-8* strain to screen 1,132 essential genes by RNAi for a cell-death defective (Ced) phenotype of the Rn.aap cells. This library of RNAi constructs targeting essential genes was compiled by a former postdoctoral fellow in the lab, Long Ma. Worms were synchronized at the first larval stage (L1) and grown in liquid culture with RNAi for five days. RNAi should gradually deplete gene activity, allowing pre-existing proteins to degrade before the Rn.aap deaths, which occur shortly before adulthood.

After four days of treatment worms reached adulthood in 813 of the 1,132 RNAi experiments, and the number of GFP-positive cells was scored for each animal as 0-4, 5-6, 7, 8-9, or 10 or more, with the latter three categories suggesting a possible Ced phenotype (Figure 5). If RNAi treatment caused Ced phenotype in greater than 8.5% of animals scored or if any animals had eight or more GFP+ cells, I selected the gene for retesting. Based on negative control experiments using an empty RNAi feeding vector, I expected a 5.2% false discovery rate by these criteria, which I deemed acceptable. Ninety RNAi constructs were selected as containing candidate Ced genes by these criteria, and RNAi treatment was repeated one to three more times, after which I identified fifty-eight candidate genes (targeted by fifty-nine RNAi constructs) (Table 1).

Analysis of candidates

Feeding of an RNAi construct that results in more than six $P_{dat\text{-}1}$*::gfp*-

expressing cells might indicate that the target gene is involved in cell death, the ray sublineage, the adoption of dopaminergic fate, the expression of the *nIs583* transgene, or other aspects of *C. elegans* biology. To distinguish among these possibilities, I repeated RNAi against these genes in worms containing a marker for the B-type neuron of the rays and in worms containing a *ced-3* mutation. RNAi that causes defects in cell death should have a normal number of B-type neurons, and perhaps enhanced numbers of A-type that should not exceed twelve. RNAi that disrupts the ray sublineage might cause alterations in the number of B-type neurons (for example, fewer if B-type neurons were transformed into A-type neurons, or more if the entire ray sublineage were repeated), and the number A-type neurons might exceed twelve in the *ced-3* strain. RNAi treatments that disrupt the adoption of dopaminergic fate in general or the expression of the transgene could also result in more than twelve GFP-postive cells in the *ced-3* strain.

I performed RNAi against the fifty-eight Ced candidates in a *ced-3* strain to determine if any caused there to be more than twelve GFP-positive cells. Twenty-eight RNAi treatments resulted in more than 12 A-type neurons in a *ced-3* background (Table 1). I performed RNAi against forty-two ced candidates in a strain containing a marker for B-type neurons in the rays, *nIs128[*$P_{pkd\text{-}2}$*::gfp]* (Barr and Sternberg, 1999; Schwartz and Horvitz, 2007). Feeding of approximately nineteen RNAi constructs caused abnormal numbers of B-type neurons (defined as fewer than 7.5 or more than 10.5, an empirically determined range). RNAi constructs that failed these tests might target genes that affect cell lineage or cell fate, but not cell death. RNAi against twenty-five genes did not strongly affect the number of B-type

neurons (or were untested) and did not enhance the number of A-type neurons in *ced-3* animals, consistent with them possibly targeting cell-death genes (marked in Table 1 with asterisks).

In parallel with this analysis, I selected four candidate genes that gave strong effects by RNAi and for which putative null alleles were available and crossed the *nIs583* reporter into mutants defective in these genes (*lpd-7, fib-1, swsn-7,* and *psf-3)*. The rays of these mutants all looked grossly normal. Only mutants of *swsn-7* had extra GFP-positive cells (88% of *swsn-7* worms had 7-9 GFP-positive cells; 0% of other mutants had more than 6 cells. *n* = 5-47). To determine if these extra GFP-positive cells were undead Rn.aap cells, I observed the cell divisions of the R5, R6, R7, R8, and/or R9 lineages in this strain. Of fourteen lineages observed in four animals, all patterns of division and cell death were normal. However, in one of these animals, there was an ectopic GFP-positive cell. Based on position, it might have been R4.aaa, which is normally a non-dopaminergic A-type neuron. Thus, the extra GFP-positive cells in animals lacking *swsn-7* might be due to an effect on the patterning of fates within the rays rather than the presence of undead Rn.aap cells.

To determine whether mutations in other candidate genes affect cell death globally, I performed the pharynx count assay in available mutants in twenty-six genes. The pharynx count assay is a sensitive, quantitative technique to score programmed cell death (Schwartz, 2007). Sixteen cells die in the anterior pharynx during embryonic development, and if any fail to die, their nuclei are visible as extra nuclei in the larval anterior pharynx. Note that this analysis occurred in parallel with the analysis described above, so some mutants were scored even if they gave

results consistent with the gene having a role in cell fate or cell lineage above. I scored 41 strains representing mutations in 24 genes but did not identify any mutants with a strong Ced phenotype by the anterior pharynx count assay (Table 2). To avoid relying on alleles with unknown impact on gene function, I used RNAi to knock down gene function in worms containing *nIs106*, a marker for the Pn.aap cells, several of which undergo programmed cell death. I performed this assay for RNAi against forty-two genes, none of which gave a striking Ced phenotype (Figure 6).

Discussion and Future Directions

The RNAi screen described above failed to reveal any new genes with a clear global role in programmed cell death. Although RNAi against several genes caused extra *nIs583*-expressing cells that repeated in quadruplicate, none of these genes played detectable roles in the ventral cord cells deaths (assayed after RNAi treatment) or the deaths in the anterior pharynx (assayed in available mutant strains). There are caveats to each of the follow-up experiments that might explain why they did not corroborate the results of the RNAi screen.

The alleles scored for candidate genes probably caused weak, if any, defects in gene activity. Because the genes analyzed are all characterized as essential genes, strong disruption of these genes would cause lethality, developmental arrest, or sterility; such mutations would not be isolated through the Million Mutation Project,

the source of many of the mutant strains used. Indeed, nonsense or missense mutations are underrepresented for essential genes in the Million Mutation Project collection (Thompson *et al.*, 2013). Most mutations I analyzed were missense alleles. Although I selected alleles that might be disruptive (*e.g.*, mutations that replace a positive amino acid with a negative one), it is unknown if the mutated residues are important for function. Furthermore, with the exception of the four candidate genes *lpd-7, fib-1, swsn-7,* and *psf-3,* the alleles were scored by the anterior pharynx assay only. A candidate gene that specifically affected the Rn.aap deaths would be expected to lack extra cells in the anterior pharynx.

The genes identified could act redundantly with *lin-35,* a gene known to promote programmed cell death (Reddien *et al.*, 2007). Although *lin-35* did not prevent cell death of the Rn.aap cells alone (Figure 3b,c), it is possible that it could cause synthetic cell death defects when a second gene's activity was reduced by RNAi. Then, during the anterior pharynx assay in mutants of candidate genes, *lin-35* function would be intact, and thus the effect of the candidate gene might have been masked. Furthermore, the cell death assay using *nIs106* to examine undead cells in the ventral cord after RNAi was done in an *rrf-3* background, not a *lin-35* background, because such a strain was readily available. However, in this background, the effect of candidate genes that work in parallel to *lin-35* would be masked as well.

To further study the possible role of these candidate genes in cell death, the anterior-pharynx and ventral-cord assays could be repeated in a *lin-35* background in case any genes might act in parallel to *lin-35* to promote cell death. Additionally,

mutations known to affect gene function should be studied. With the recent development of CRISPR/Cas9 genome editing techniques, it might be possible to generate stronger alleles for these genes. It is also possible that for some genes, the RNAi effects are the result of off-target effects, not knock-down of the intended gene.

In addition to the candidate genes that I identified, there might be other essential genes with a role in cell death that were missed. More than a quarter of RNAi treatments caused lethality or larval arrest and could not be scored. Ninety-five more RNAi experiments had fewer than ten male adults that could be scored, so small effects on cell death would be likely overlooked. Furthermore, it is likely that the annotated identity of the RNAi clone is wrong in some cases; we sequenced ten RNAi constructs and found that two were annotated as the incorrect gene. For these reasons, the lack of a Ced phenotype in my screen should not be interpreted to mean that the gene in question does not play a role in cell death. In addition, the number of essential genes is likely 3,000-6,000, so this screen was not comprehensive (Kemphues, 2005).

Materials and Methods

Strains and Genetics

C. elegans was maintained on nematode growth medium (NGM) Petri plates at 20°C (Brenner, 1974). *him-8(e1489)* and *him-5(e1490)* were to generate a high incidence of males, which facilitated study of the male-specific cells of the ray lineages (Hodgkin, 1983). The following mutations, integrations, and extrachromosomal

arrays were used: LGI: *psf-3(ok2828)*, *lin-35(n745)*, *nIs584[P_{dat-1}::4xNLS::gfp::unc-54 3' UTR, P_{myo-2}::mCherry::unc-54 3' UTR]* (this study). LGII: *rrf-3(pk1426)*, *swsn-7(gk1041)*, *nIs128[P_{pkd-2}:: gfp, lin-15(+)].* LGIII: *hpl-2(ok917)*, *lpd-7(gk78910)*, *lpd-7(ok870)*, *nIs583[P_{dat-1}::4xNLS::gfp, P_{myo-2}::mCherry::unc-54 3' UTR]* (this study). LGIV: *him-8(e1489)*, *ced-3(n2427)*, *ced-3(n717).* LGV: *him-5(e1490)*, *fib-1(ok2527)*, *fib-1(gk565835)*, *fib-1(gk675991).* LGX: *nre-1(hd20)*, *lin-15B(hd126)*, *nIs116[P_{cat-2}::gfp, lin-15(+)]*, *nIs106[P_{lin-11}::gfp, lin-15(+)]*. Unmapped integrants: *nIs143[P_{dat-1}::dat-1::gfp, rol-6(su1006)].* Where unspecified, the *n717* allele of *ced-3* was used.

General microscopy.

For quantification of ray cells in strains without RNAi treatment and for quantification of cells in the anterior pharynx, I mounted animals in 20-60 mM sodium azide on 4% agar pads and visualized worms using Nomarski differential interference contrast optics. Determination of ray cell lineage was performed as described (Sulston and Horvitz, 1977). Microscopy was done using a fluorescence-equipped Axioskop II (Zeiss) compound microscope. Images were acquired with an ORCA-ER CCD camera (Hamamatsu) using OpenLab software (Agilent).

To score GFP-positive cells in RNAi conditions, sodium azide was added to a final concentration of about 60 mM to worms in flat-bottomed 96-well plates. Animals were visualized using an inverted Nikon TE-2000 compound microscope equipped for fluorescence microscopy (Prior Scientific).

To score survival of Pn.aap cells, free-moving worms on NGM RNAi plates were visualized using an Olympus SZX12 dissecting scope equipped for florescence microscopy.

Transgene construction.

To create the $P_{dat\text{-}1}$*::gfp::unc-54 3' UTR* transgene, a 716 bp fragment 5' of *dat-1* was PCR amplified using primers with *Pst*I and *Hind*III restriction sites incorporated on the 5' ends to facilitate cloning. The purified PCR amplicon and plasmid pPD122.56 were digested with *Hind*III and *Pst*I and the purified digestion products were ligated together. The plasmid was injected at 150 ng/µL into *him-8(e1489) ced-3(n717)* animals with 2.5 ng/µL of pCFJ90[$P_{myo\text{-}2}$*::mCherry::unc-54 3' UTR*] as a co-injection marker. Animals containing the construct as an extrachromosomal array were irradiated with a dose of approximately 4,600 Rads using a gamma irradiator to induce genomic integration.

RNA-interference

For the RNAi screen, *lin-35 ; nIs583; him-8* gravid animals were bleached in 4.4% NaHOCl with 0.7 M NaOH for 10 minutes with periodic vortexing to recover eggs. Eggs were hatched in M9 solution without food overnight to synchronize worms in the first larval stage. These animals were diluted to approximately 50 per 20 µL and used to set up RNAi in liquid culture in 96-well plates as described (Ahringer, 2006)

96 well plates were incubated at 20°C in a humid chamber with gentle shaking for five days before scoring.

For analysis of Pn.aap survival experiments, 5 fourth-larval stage *rrf-3; nIs106* hermaphrodites were placed on RNAi plates and progeny were scored 3-5 days later in adulthood. RNAi plates were made by adding 50 μg/mL carbenicillin, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM IPTG, and 1/40 dilution of PO_4 to standard NGM. These plates were spotted with 100 μL of an overnight bacterial colony and incubated at 37°C for at least twelve hours before using.

False-discovery rate estimation

To estimate the false-discovery rate, an expected percentage of false-positives, I performed RNAi using the empty RNAi vector L440 in ninety-six replicates. Each well contained between three and thirty animals. From this experiment, five wells met the criteria of having more than 8.5% of animals with seven or more GFP-positive cells (four wells) or containing an animal with eight or more GFP-positive cells (one well).

Cell death assays

Cell death assays were performed as previously described (Reddien *et al.*, 2001; Schwartz, 2007).

Acknowledgments

I am grateful to Peter Reddien for the inspiration for this project. I thank Long Ma for building the essential gene RNAi library and Rita for help validating clone sequences. Thank you to Vivek Dwivedi and my thesis committee for comments on this chapter.

Figure 1: The ray sublineage. Adapted from (Portman and Emmons, 2004). Nine Rn ray precursors on the left and right sides of the developing male tail undergo the same pattern of cell divisions to generate nine bilateral pairs of rays, each composed of two neurons and a structural cell. A hypodermal cell and a cell that dies are also generated in each Rn sublineage.

Figure 1:

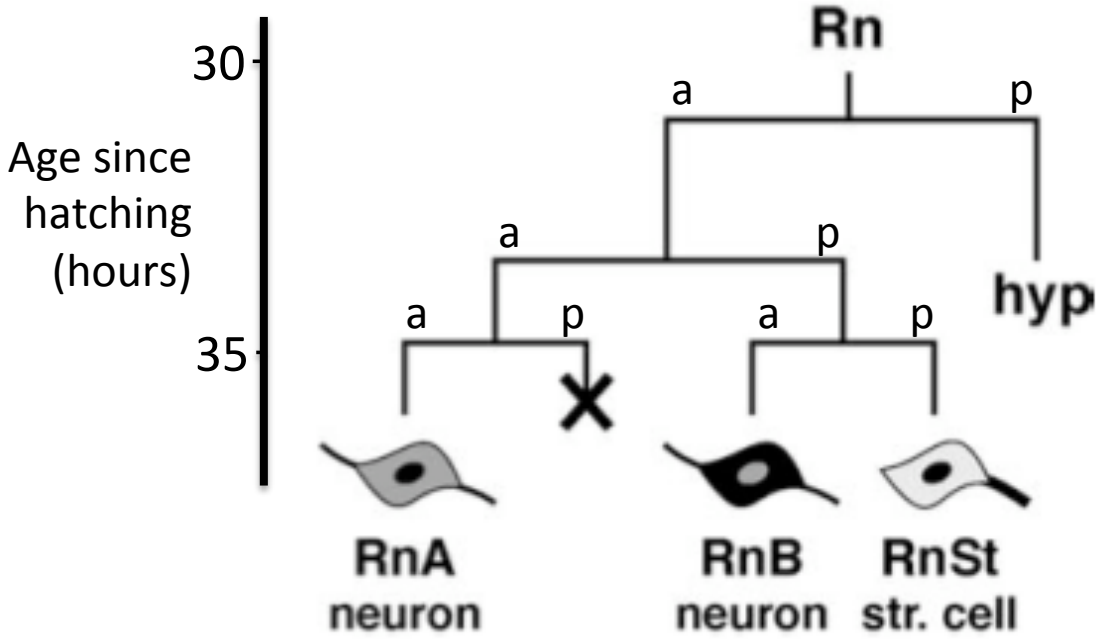
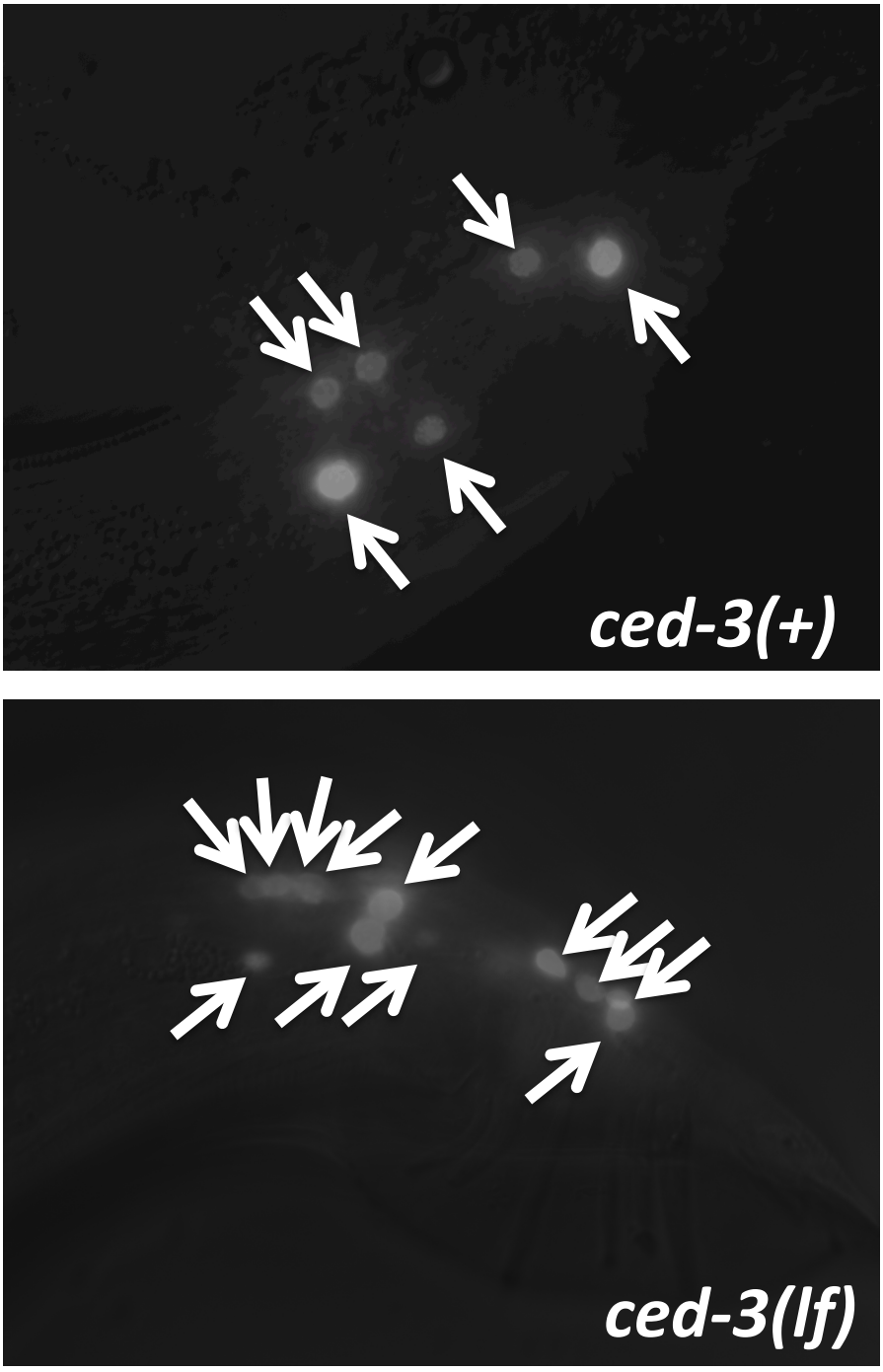

Rn
30
Age since hatching (hours)
35
a
p
a
p
a
p
a
p
hyp
RnA neuron
RnB neuron
RnSt str. cell

Figure 2: **Expression of *$P_{dat\text{-}1}$::4xNLS::gfp***. The integrated transgene *nIs583* causes GFP to be expressed in the R5A, R7A, R9A cells (*i.e.* the A-type neuron of the R5, R7, and R9 sublineages). In animals wild-type for cell death genes, there are usually six GFP-positive cells in the adult male tail. In *ced-3* mutant animals, there are extra GFP-positive cells, presumably the undead Rn.aap cells of the R5, R7, and R9 sublineages. The number of extra Rn.aap cells depends on the strength of the *ced-3* mutant allele analyzed; the strong *ced*-3 mutation *n717* typically gives 4-6 extra GFP-positive cells (*n* = 95), while the weaker allele *n2427* typically gives 1-3 extra GFP-positive cells (*n* = 54).

Figure 2:

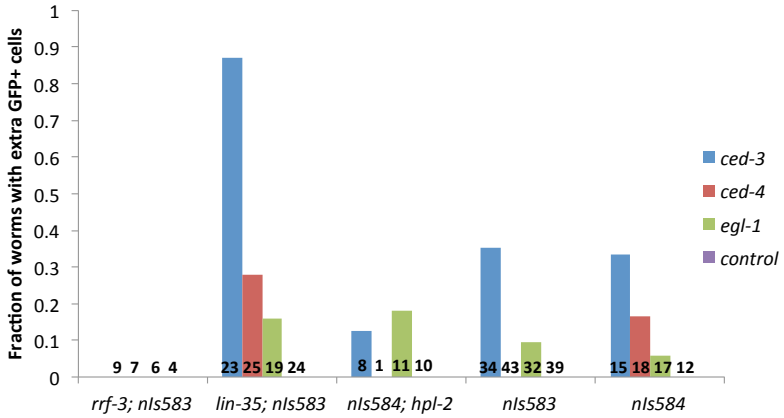


Both strains contain *lin-35; nIs583; him-8*

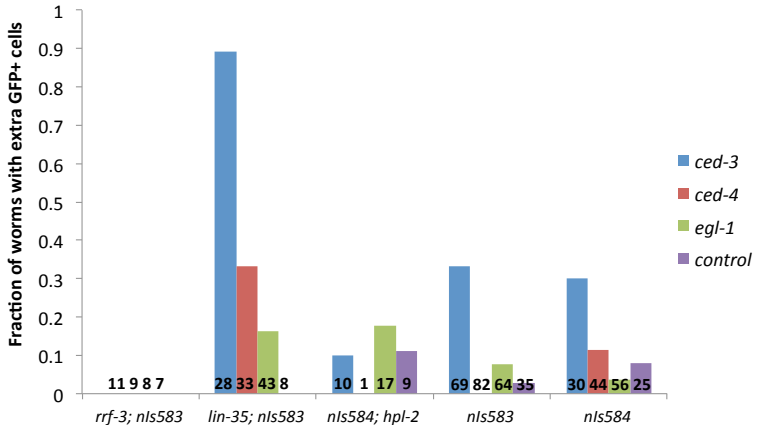
Figure 3: Testing of RNAi sensitive strains. a-c) Data are shown for three days of experimentation. The *rrf-3; nIs583* strain had silencing of the *nIs583* transgene and had few GFP-expressing cells, even when cell-death genes were targeted (a,b). RNAi against *ced* genes generally caused an enhanced Ced phenotype (greater than 6 GFP-positive cells) in *nIs583; lin-35* animals compared to animals of other genotypes. Control conditions, with feeding RNAi using an empty RNAi vector, did not cause a Ced phenotype in the *nIs583; lin-35* strain (a,b,c). RNAi against *ced-3* and *ced-4* had less robust effect in the *nIs584; hpl-2* strain (a,b,c). RNAi in non-sensitized strains (*nIs583* and *nIs584*) had moderate sensitivity to RNAi against cell-death genes (a,b,c) RNAi against *ced-3* and *egl-1* had *nIs583; nre-1; lin-15B* animals gave a slightly weaker Ced phenotype than in *nIs583; lin-35* animals, but had a slightly higher backround level of Ced animals in the negative control RNAi condition (c). Control conditions, with feeding RNAi using an empty RNAi vector, unexpectedly had a high number of animals displaying the Ced phenotype in the non-sensitized strain (*nIs583*) on the third day (c). This phenotype was most likely caused by contamination of RNAi bacteria between wells, as this was not seen on other days. RNAi treatments in the *nIs583 lin-35* strain most consistently gave a Ced phenotype in response to RNAi knockdown of cell-death genes, while control RNAi conditions had a low level of background Ced worms, so it was selected for use in the screen.

Figure 3:

a) 7/26/12



b) 8/6/12



c) 9/14/12

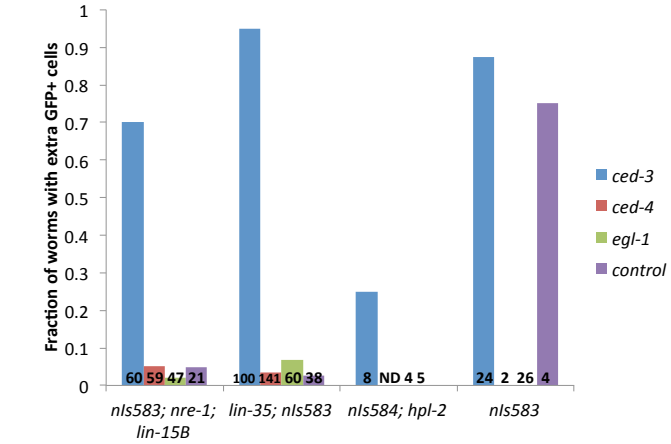


Figure 4: Standard IPTG concentration gives more robust RNAi knockdown of *ced-3.* An IPTG concentration of 1 mM gave a stronger Ced phenotype in four of five genotypes than did a concentration of 2.5 mM. *n* = 8-34

Figure 4:

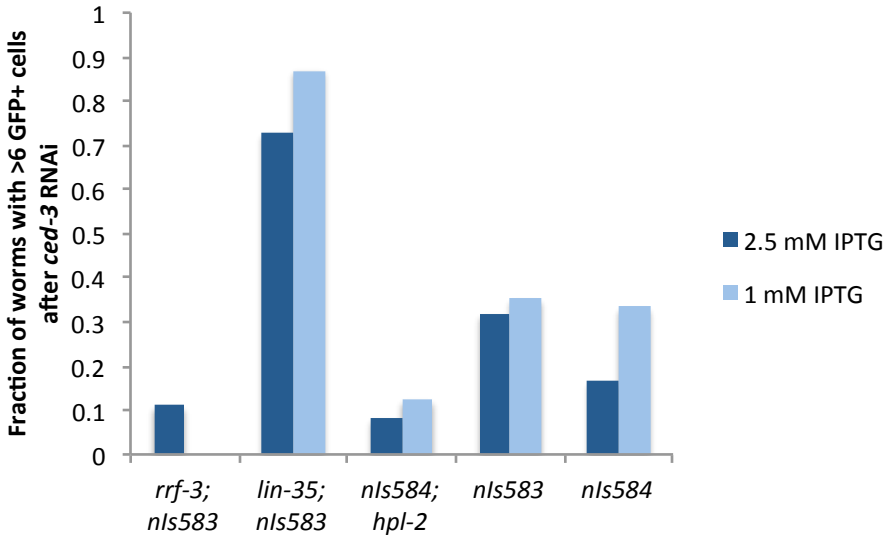

Fraction of worms with >6 GFP+ cells after ced-3 RNAi
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
rrf-3; nIs583
lin-35; nIs583
nIs584; hpl-2
nIs583
nIs584
2.5 mM IPTG
1 mM IPTG

Figure 5: Overview of phenotypes scored in RNAi screen. Worms containing extra GFP-positive cells (red) were observed after feeding of 220 of 813 RNAi constructs tested. Many RNAi treatments also reduced the number of GFP-expressing cells in some animals(blue), perhaps by disrupting development generally. The black horizontal line approximately marks 8.5%, the threshold percentage of worms with extra GFP-positive cells to be retested. *n* = 1-63 for each condition (21 animals on average)

Figure 5:

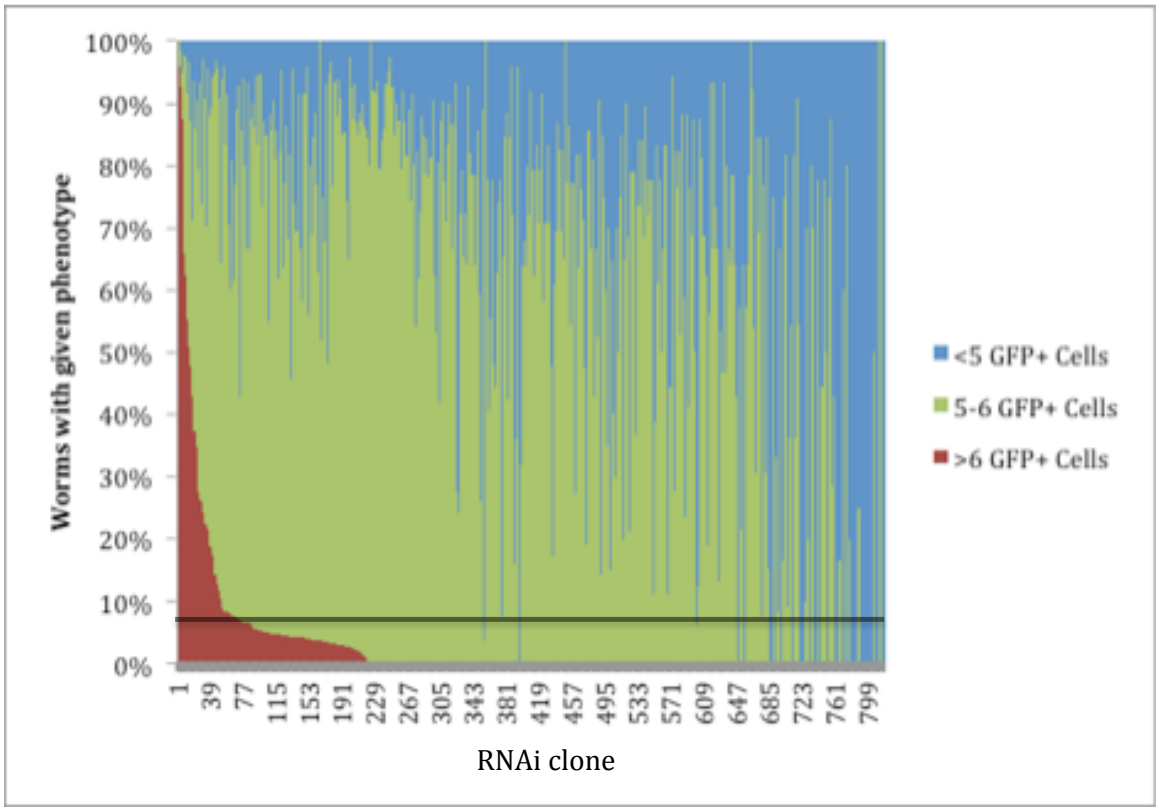

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Worms with given phenotype
1
39
77
115
153
191
229
267
305
343
381
419
457
495
533
571
609
647
685
723
761
799
RNAi clone
<5 GFP+ Cells
5-6 GFP+ Cells
>6 GFP+ Cells

Figure 6: Quantification of surviving Pn.aap cells in *nIs106*. After RNAi treatment against the specified gene in *rrf-3; nIs106* animals, the number of GFP-positive cells were counted (excluding two near the vulva, which is also GFP-positive, making these hard to score). A non-cell death defective animal has four GFP-positive cells by this assay; RNAi against *ced-3* resulted in up to six extra cells. Error bars represent standard deviation. *n* = 2-58.

Figure 6:

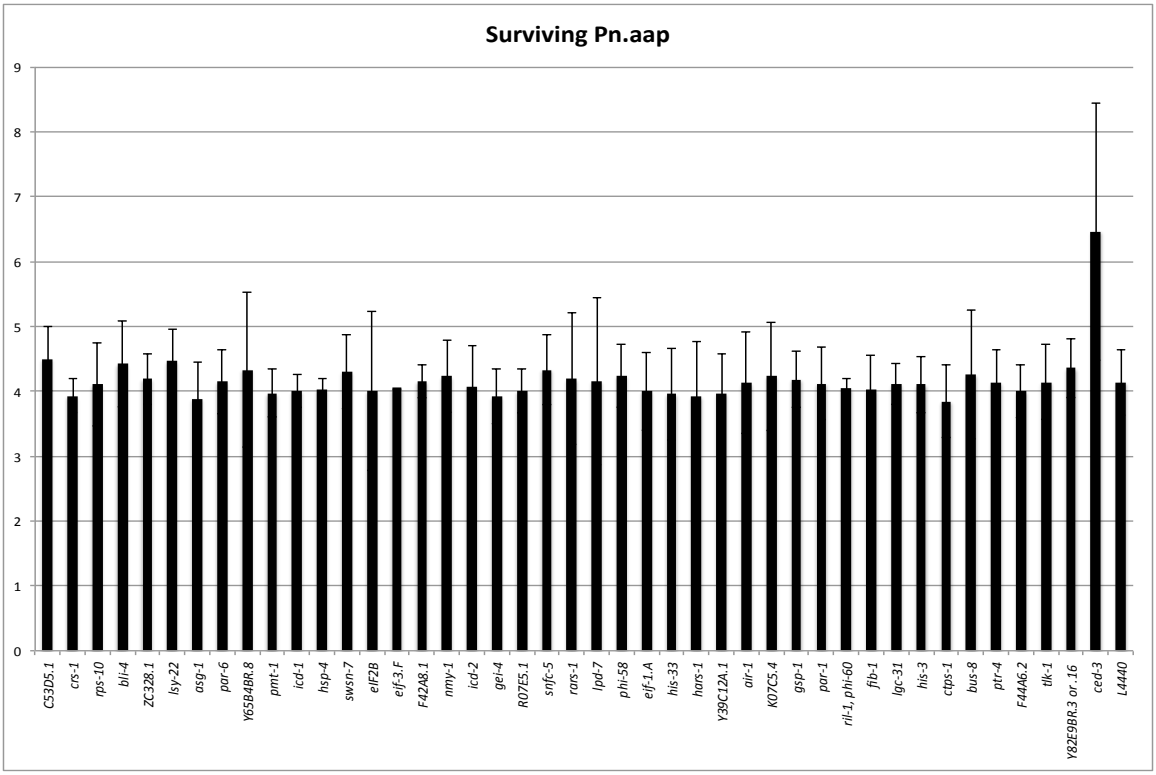

Surviving Pn.aap
9
8
7
6
5
4
3
2
1
0
C53D5.1
crs-1
rps-10
bli-4
ZC328.1
lsy-22
asg-1
par-6
Y65B4BR.8
pmt-1
icd-1
hsp-4
swsn-7
eIF2B
eif-3.F
F42A8.1
nmy-1
icd-2
gei-4
R07E5.1
snfc-5
rars-1
lpd-7
phi-58
eif-1.A
his-33
hars-1
Y39C12A.1
air-1
K07C5.4
gsp-1
par-1
ril-1, phi-60
fib-1
lgc-31
his-3
ctps-1
bus-8
ptr-4
F44A6.2
tlk-1
Y82E9BR.3 or .16
ced-3
L4440

Table 1: **Fifty-eight candidate genes, RNAi treatment of which caused the appearance of extra GFP-positive cells in the male tail.** List of genes is sorted by fraction of worms with extra GFP-positive cells. Note that two RNAi constructs, number 18 and 23 of this list, were found to target the same gene. RNAi constructs that caused greater than 8.5% of worms to have extra cells, or that caused any worms to have two or more extra cells, were considered candidates. These RNAi constructs were further tested to determine if RNAi in a *ced-3(n717)* background could ever result in thirteen or more GFP-positive cells in the male tail; this fraction is given in the table. Pink highlights non-zero values of this fraction. These RNAi constructs were also tested for their ability to affect the number of B-type neurons, marked by *nIs128*. Red color corresponds to a more abnormal number of B-type neurons. ND: not determined. RNAi treatments that did not cause greater than 13 GFP-positive cells in *ced-3* or greatly affect the number of B-type neurons are highlighted by bold text and an asterisk.

Table 1:

	RNAi clone	Gene targeted	Overall fraction animals with >6 GFP+ cells	Max. GFP+ cells in one animal	*N*	Fraction worms with >13 cells after RNAi in *ced-3* background	Count of *nIs128* (B-type neuron marker)
1	*K10B2.1*	*lin-23*	1.000	many	36	1.00	8.6
2	*T28F12.2a*	*unc-62*	1.000	many	18	1.00	ND
3	*D2045.6*	*cul-1/lin-19*	0.960	many	25	1.00	8.2
4	*F32H2.5*	*fasn-1*	0.927	many	41	1.00	9.6
5	*ZK1236.3*	*sor-1*	0.900	many	40	0.93	12.1
6	*ZK858.4*	*mel-26*	0.875	many	56	0.89	10.6
Positive control	*ced-3*	*ced-3*	0.842	12	139	0.00	9.3
7	*W07B3.2*	*gei-4*	0.796	many	54	1.00	10.8
8	*R06F6.1*	*cdl-1*	0.659	12	44	0.19	4.6
9	*E03H4.8*	*F33H2.5?*	0.625	14	40	0.44	11.1
10	*C08B11.3*	*swsn-7*	0.602	9	103	0.06	14.1
11	*K02F2.3*	*teg-4*	0.553	10	47	0.20	9.3
12	*C04A2.2*	*C04A2.2*	0.512	11	41	0.33	11.5
13	*Y105E8C.d*	*psf-3*	0.509	9	116	0.01	8.4
14*	***C06B8.8***	***lgc-31***	**0.506**	**12**	**79**	**0.00**	**9.3**
15	*C07A9.3*	*tlk-1*	0.479	10	71	0.02	3.8
16	*C36B1.5*	*prp-4*	0.468	many	47	0.62	12.6
17*	***T01C3.7***	***fib-1***	**0.429**	**9**	**14**	**0.00**	**ND**
18	*R13A5.12*	*lpd-7*	0.413	10	46	0.08	8.3
19*	***D2013.7***	***eif-3.F***	**0.374**	**10**	**99**	**0.00**	**8.0**
20	*F47D12.4*	*hmg-1.2*	0.372	10	43	0.52	12.0
21*	***C56C10.8***	***icd-1***	**0.370**	**11**	**73**	**0.00**	**9.1**
22	*F11H8.4*	*cyk-1*	0.348	9	46	0.05	10.3
23*	***R13A5.13***	***lpd-7***	**0.282**	**9**	**78**	**0.00**	**8.5**
24*	***Y51H7B_5.b***	***icd-2***	**0.275**	**9**	**80**	**0.00**	**7.9**
25*	***F42A8.1***	***F42A8.1***	**0.272**	**11**	**114**	**0.00**	**7.5**
26	*W06H3.3*	*ctps-1*	0.263	12	76	0.02	9.9
27	*F22D6.3*	*nars-1*	0.262	13	42	0.11	13.0
28*	***T07A9.9***	***phi-58***	**0.260**	**9**	**50**	**0.00**	**ND**
29	*C09D4.5*	*rpl-19*	0.250	8	32	0.10	10.5
30*	***F26F4.10***	***rars-1***	**0.243**	**10**	**37**	**0.00**	**ND**
31*	***F17E9.13***	***his-33***	**0.227**	**8**	**22**	**0.00**	**ND**
32	*T11G6.1*	*hars-1*	0.225	10	71	0.00	4.5
33	*W06F12.1*	*lit-1*	0.222	12	27	0.05	4.3
34	*R12E2.3*	*rpn-8*	0.217	7	46	0.05	12.7

35*	***ZK622.3***	***pmt-1***	**0.214**	**9**	**140**	**0.00**	**8.1**
36*	***T10C6.12***	***his-3***	**0.200**	**7**	**25**	**0.00**	**ND**
37	*K04F10.4*	*bli-4*	0.190	10	100	0.00	2.7
38*	***T23F2.1***	***bus-8***	**0.188**	**10**	**48**	**0.00**	**ND**
39*	***Y23H5A.7***	***crs-1***	**0.187**	**8**	**75**	**0.00**	**ND**
40	*F52B11.3*	*noah-2*	0.182	8	22	0.07	8.3
41	*Y119D3_446.a*	*Y82E9BR.3 or Y82E9BR.16*	0.174	9	69	0.00	7.0
42	*F27D4.2*	*lsy-22*	0.170	8	100	0.03	ND
43	*C45B2.7*	*ptr-4*	0.143	8	63	0.00	6.8
44*	***C53A5.1***	***ril-1, phi-60***	**0.143**	**9**	**154**	**0.00**	**ND**
45*	***H06H21.3***	***eif-1.A***	**0.129**	**8**	**85**	**0.00**	**8.5**
46	*W01G7.2*	*nmy-1*	0.127	8	118	0.00	11.7
47*	***R07E5.1***	***R07E5.1***	**0.122**	**8**	**98**	**0.00**	**ND**
48	*ZC250.3*	*nstp-3*	0.118	8	85	0.09	ND
49*	***K07C5.4***	***K07C5.4***	**0.109**	**9**	**101**	**0.00**	**ND**
50	*F29F11.6*	*gsp-1*	0.104	8	134	0.03	ND
51	*F33D11.10*	*F33D11.10*	0.089	9	56	0.07	ND
52*	***H39E23.1***	***par-1***	**0.088**	**9**	**114**	**0.00**	**8.4**
53*	***C53D5.a***	***C53D5.1***	**0.082**	**8**	**61**	**0.00**	**ND**
54	*Y39C12A.a*	*Y39C12A.1*	0.078	9	90	0.00	7.1
55*	***K07A12.3***	***asg-1***	**0.067**	**12**	**119**	**0.00**	**ND**
56*	***R07E5.3***	***snfc-5***	**0.065**	**8**	**108**	**0.00**	**8.2**
57*	***F44A6.2***	***F44A6.2***	**0.060**	**9**	**100**	**0.00**	**ND**
58*	***D2085.3***	***eIF2B***	**0.044**	**8**	**137**	**0.00**	**9.0**
59*	***K07C11.2***	***air-1***	**0.043**	**8**	**138**	**0.00**	**ND**
Negative control	L4440	none	0.015	8	1538	0.00	9.1

Table 2: Pharynx counts in candidate mutants. The number of extra cells in the anterior pharynx were counted in strains that contained mutations for candidate genes from the RNAi screen. No strains greatly affect the survival of cells in the anterior pharynx. Alleles of *ced-3* are included as positive controls. S.E.M.: standard error of the mean.

Table 2:

Strain	Gene mutated	Average extra cells in pharynx	S.E.M.	*N*
	ced-3(n2427)	2.60	0.52	12
	ced-3(n717)	9.40	1.02	7
CB937	*bli-4*	0.00	0.10	15
VC20291	*C53D5.1*	0.00	0.00	15
VC20165	*C53D5.1*	0.07	0.07	15
VC40755	*crs-1*	-0.36	0.17	14
VC110	*ctps-1*	0.13	0.09	15
VC40785	*ctps-1*	0.13	0.09	15
VC20030	*eif-1A*	0.07	0.07	15
VC30195	*eif-1A*	0.20	0.11	15
VC30127	*eif-1A*	0.80	0.22	15
VC20097	*eif-3.F*	0.07	0.07	15
VC40587	*eif-3.F*	0.66	0.19	15
VC40177	*eIF2B*	0.13	0.09	15
VC40640	*eIF2B*	0.27	0.12	15
VC40817	*eIF2B*	0.25	0.17	16
VC20419	*F42A8.1*	0.07	0.07	15
CB4856	*F42A8.1*	0.14	0.14	14
RB1960	*F42A8.1*	-0.27	0.20	15
VC40518	*fib-1(gk675991)*	0.27	0.12	15
VC40307	*fib-1(gk565835)*	0.73	0.21	15
VC40745	*hars-1*	0.40	0.13	15

VC1099	*hsp-4*	0.20	0.11	15
VC40979	*lgc-31*	0.07	0.15	15
VC40795	*lgc-31*	0.47	0.19	15
VC40190	*lgc-31*	0.53	0.21	15
VC40740	*lpd-7(gk787910)*	0.40	0.16	15
HR1184	*nmy-1*	0.13	0.09	15
FT36	*par-6*	0.13	0.09	15
VC40091	*phi-58*	0.00	0.00	15
VC20231	*phi-58*	0.07	0.12	15
VC40321	*phi-58*	0.40	0.13	15
VC40853	*pmt-1*	0.07	0.07	15
VC20709	*rps-10*	0.20	0.14	15
RB810	*snfc-5*	0.06	0.05	16
VC2538	*swsn-7(gk1041 lf)*	0.25	0.16	8
JS604	*tlk-1*	0.45	0.16	11
VC40422	*Y39C12A.1*	0.00	0.00	15
VC20527	*Y39C12A.1*	0.07	0.07	15
VC20272	*Y39C12A.1*	0.13	0.09	15
VC40970	*Y39C12A.1*	0.13	0.09	15
VC2130	*psf-3(ok2828 lf)*	0.27	0.25	15
VC30130	*Y82E9BR.16*	0.07	0.12	15

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Chapter 4

Cell-death gene expression as detected by single molecule fluorescent *in situ* hybridization

Summary

It has been proposed that all cells in *C. elegans* might express cell death genes (Shaham and Horvitz, 1996) and that the regulation of *egl-1* expression is a critical step in initiating programmed cell death (Nehme and Conradt, 2009). However, the endogenous patterns of expression of the cell-death genes are still unknown. In this chapter, I describe the gene expression patterns of the cell-death genes *egl-1, ced-9, ced-4,* and *ced-3* based on single molecule fluorescent *in situ* hybridization (smFISH) experiments, a technique that allows visualization of single molecules of mRNA (Raj *et al.,* 2008).

Introduction

Programmed cell death occurs during the normal development of many organisms (Ellis *et al.,* 1991). The *C. elegans* cell-death pathway has been extensively studied for a variety of cells that are fated to die. During programmed cell death, the BH3-only protein EGL-1 is produced, which binds to CED-9 and causes it to release CED-4 (Conradt and Horvitz, 1998; Hengartner *et al.,* 1992; del Peso *et al.,* 1998; Yuan and Horvitz, 1992). Released CED-4 forms an octomeric apoptosome that binds to CED-3 precursors and promotes their cleavage and activation (Huang *et al.,* 2013; Yang *et al.,* 1998). CED-3 is a caspase that can cleave many targets in the cell to cause cell death (Taylor *et al.,* 2007; Yuan *et al.,* 1993).

This model suggests that CED-9, CED-4, and CED-3 protein are present in cells before they are specified to die, but that CED-3 is typically present in an

inactive form and CED-4 is sequestered by CED-9. However, the expression of these genes during the larval stages has primarily been analyzed using northern blots to detect mRNA levels in whole worm lysis or by visualizing GFP-tagged transgenes (Chen *et al.,* 2000; Hengartner and Horvitz, 1994; Yuan and Horvitz, 1992; Yuan *et al.,* 1993). Although it is assumed that *egl-1* is transcribed only in dying cells, our understanding of the transcription patterns of *egl-1* and *ced-3* are based on the expression of transgenes, which might not represent the endogenous patterns of gene expression, in part because of difficulty creating antibodies against some of the cell death proteins (Hirose *et al.,* 2010; Maurer *et al.,* 2007; Nehme *et al.,* 2010). To my knowledge, the spatial distribution of *ced-4* or *ced-9* gene expression has not been characterized in larvae.

Single molecule fluorescent *in situ* hybridization (smFISH) is a technique that allows for the detection of single mRNA transcripts (Raj *et al.,* 2008). For this technique, 21-48 probes are designed to bind in a tiling fashion along a strand of mRNA. Since each of the many probes binds along an individual transcript, the fluorescent molecules attached to each of the probes are concentrated in a small area and become visible as a diffraction-limited spot of fluorescence. No secondary amplification of the signal is necessary, and this technique is assumed to be highly specific, as many probes must be complementary to a strand of mRNA before their fluorescence becomes detectable.

To determine the spatial pattern of cell death genes in *C. elegans* larvae, I performed smFISH to determine when and where *egl-1*, *ced-9*, *ced-4*, and *ced-3* were expressed.

Results

In order to analyze worms at various larval stages, I synchronized worms by hatching eggs without food overnight. Animals that hatch without food will arrest development until food is present. I selected four time points during development for analysis, which correspond to approximately 8.3, 15.5, 18, and 28 hours after hatching. These time points were chosen so that fixed worms might have some dying, dividing, and postmitotic cells (Figure 1). Additionally, I analyzed the male tail during the fourth larval stage when many cell divisions and several cell deaths occur.

First larval stage

First-larval stage animals aged approximately 8.3 or 15.5 hours after hatching were fixed. 8.3 hours is near the age that cell deaths in the Q lineage (QL.aa, QR.aa, QL.pp, QR.pp) occur and progeny of the somatic blast cells H, T, W, M, Q, Z, and P divide (Sulston and Horvitz, 1977). I found that *ced-9* and *ced-4* were both broadly expressed throughout the animal (Figure 2). *egl-1* was expressed in small clusters consistent with transcription occurring only in specific cells (Figure 2c). These might be dying cells in the Q lineage, but their identities are not known. *ced-3* transcripts were detectable in the germline as well as sparsely in areas of the worm (Figure 2a,b).

At 15.5 hours, animals are near the age when some deaths in the P cell lineage occur (specifically, the P12.pp, P12.aap, P11.aaap, and P12.aaap cell deaths in the posterior ventral cord), and the progeny of blast cells M, P, H, and V divide

(Sulston and Horvitz, 1977). As in younger worms, *ced-9* and *ced-4* were broadly expressed (Figure 3). *egl-1* was expressed in a limited number of cells, but these were not limited to the posterior ventral cord, where dying cells were expected to be located (Figure 3a,b). There were also *egl-1* expressing cells in the head and anterior body (Figure 3a,b). These might be the dying cells P0.aap, P1.aap, or P2.aap, which are located in the anterior ventral cord and die around 11-12.5 hours after hatching. Many animals had sparse *ced-3* transcripts throughout the worm (Figure 3c), and one animal also had multiple *ced-3* transcripts clustered in each of what appeared to be three cells in the posterior ventral cord (Figure 3d).

Second larval stage

Second-larval stage animals aged approximately 18 hours after hatching were fixed. 18 hour-old animals were not expected to have any dying cells (Sulston and Horvitz, 1977). Somatic cells of the V, Z, T, and G2 lineages were expected to be dividing (Sulston and Horvitz, 1977). As in the first larval stage, *ced-4* and *ced-9* were broadly expressed (Figure 4). Although dying cells were not expected, *egl-1* transcripts were concentrated in variable cells in the head, mid-body, posterior, and tail (Figure 4a,b). At least some of these are likely of the P11 or P12 lineages (for example, P12.aaap, P11.aaap, P12.aap, or P12.pp), because they are located along the ventral cord in locations where deaths typically occur (Sulston and Horvitz, 1977). *ced-3* transcripts were visible in the germline and also sparsely throughout the worm (Figure 4c,d).

Third larval stage

Third-larval stage animals aged approximately 28 hours after hatching were expected to contain no dying cells (Sulston and Horvitz, 1977). Some somatic cells of the Z, M, and P lineages were expected to be dividing (Sulston and Horvitz, 1977). As in the previous larval stages, *ced-4* and *ced-9* were broadly expressed (Figure 5). Although no dying cells were expected to be present in animals of this age, *egl-1* transcripts were detectable in a small number of cells in each animal analyzed (Figure 5e,f). It is possible that some might be cell corpses that have not been fully degraded. Indeed, some are located in positions consistent with earlier deaths, such as the postdeirid (Sulston and Horvitz, 1977). Also, based on the size of the germline (Kimble and Hirsh, 1979), it is likely that the worms analyzed were slightly younger than expected, so there might actually be dying cells present. However, others are located in areas where no larval cell deaths occur. For example, occasionally cells in the head contained *egl-1* (Figure 5e); these might be neurons. *ced-3* transcripts were visible in the germline and also sparsely throughout the worm (Figure 5a,c).

Fourth-larval stage male tail

Fourth-larval stage animals aged approximately 34-37 hours after hatching were fixed for analysis of the developing male tail. During this period, cells of the Rn sublineages (nine each on the left and right side of the tail) divide to create the rays (Sulston and Horvitz, 1977). Nine Rn.aap cells on the left and on the right undergo programmed cell death as do five cells of the B lineage, B.alapaav or B.arapaav, B.γald or B.γard, B.alappd, B.arappd, and B.ppaa (Sulston *et al.,* 1980). The linker cell also dies later in the fourth-larval stage (Kimble and Hirsh, 1979; Sulston *et al.,* 1980). As in other tissues and stages, *ced-4* and *ced-9* are expressed broadly in the

male tail (See Chapter 2, Figure 4). *ced-3* transcript levels seem to be dynamic; in some animals *ced-3* transcripts are fairly broadly expressed in what seem to be newly-created cells of the B and Rn lineages. However in other animals, *ced-3* localization is limited to the germline, cells that appear to be in the ray sublineages, and cells in positions consistent with being the dying cells of the B lineage (Figure 6). In a few older animals, I detected *ced-3* transcripts in a cell that was likely the linker cell (Figure 7), a cell that undergoes a caspase-independent, non-apoptotic programmed cell death (Abraham *et al.,* 2007; Blum *et al.,* 2012; Denning *et al.,* 2013).

Discussion

The observations described above are preliminary and are based on a small number of worms (n = 4-34). Because no markers for specific cell fates were used, the identity of individual cells can only be hypothesized based on their positions. Therefore, the conclusions that can be drawn from this study should be considered tentative.

In all larval stages analyzed, I found that *ced-9* and *ced-4* are broadly expressed throughout the animal. It has been previously reported that *ced-9* mRNA transcripts are present in all larval stages (Hengartner and Horvitz, 1994), but *ced-4* mRNA is only barely detectable in larval animals by northern blot (Yuan and Horvitz, 1992). In embryos, CED-9 and CED-4 are also broadly expressed in most if not all cells, as measured by antibody staining (Chen *et al.,* 2000). The same researchers report that neither CED-9 nor CED-4 was present in larvae or adults.

However, larval cell deaths require *ced-9* and *ced-4* function (Ellis and Horvitz, 1986; Hengartner *et al.,* 1992) so it is unlikely that these proteins are truly absent from larvae. It is likely that antibody staining is unable to detect low levels of protein, but smFISH, which can detect single molecules of RNA, is a highly sensitive way to analyze gene expression. Based on the requirement for *ced-9* and *ced-4* for larval cell deaths and the presence of *ced-9* and *ced-4* mRNA throughout larval worms of all stages analyzed, I predict that at least small amounts of CED-9 and CED-4 protein are present in most if not all cells.

ced-3 mRNA transcripts were highly expressed in the germline in all larval stages analyzed. This finding is consistent with the report that *ced-3* RNA is expressed in all larval stages, but that *glp-1* adults, which lack a germline, had no detectable *ced-3* RNA (Yuan *et al.,* 1993). Cell-specific *ced-3* upregulation directly before cell death has only previously been reported for two types of cells: the tail-spike cell and the CEM cells in the hermaphrodite (Maurer *et al.,* 2007; Nehme *et al.,* 2010). However, *ced-3* appears to be transcribed in specific cells fated to die before their deaths in the fourth larval stage male tail as well. The tail-spike cell and CEM cells both live for over two hours before dying (Sulston *et al.,* 1983). It has been proposed that other cells that die might inherit pro-CED-3 during cell division that is sufficient to cause cell death, but that any inherited pro-CED-3 might have already degraded before the late cell deaths in the tail-spike or CEM cells, requiring new gene expression (Maurer *et al.,* 2007; Nehme *et al.,* 2010). Some of the dying cells in the male tail are also long lived, such as B.al/rapaav, B.γal/rd, and the linker cell; each lives at least five hours before dying (Sulston *et al.,* 1980). Interestingly, I found

that these cells each seem to have cell-specific *ced-3* transcription before their deaths. Thus, most if not all long-lived cells in *C. elegans* might need an extra burst of *ced-3* gene expression before programmed cell death.

In many of the older-staged fixations, the progress of the migration of the germline and the locations of *egl-1* expression were inconsistent with the expected age; animals appeared younger than expected based on the timing of the fixation. It is possible that there is a delay in development of several hours after food-deprived animals are placed onto food. If this is taken into account, the expression of *egl-1* transcribing cells is more consistent with that of known locations and timings of cell deaths that occur during larval development. *egl-1* mRNA transcripts were detected in cells in positions consistent with dying cells, supporting the model that *egl-1* transcription is generally tightly controlled and sufficient for inducing programmed cell death (Nehme and Conradt, 2009). However, *egl-1* transcripts were also occasionally found in other cells that are unlikely to be programmed to die, such as cells in the head. Perhaps *egl-1* transcription begins in in these cells after inherited pro-CED-3 has been degraded, so the production of EGL-1 is insufficient to induce cell death in these cells. Alternatively, low levels of *egl-1* expression might be insufficient to induce cell death in some cells. This insufficiency could be because *egl-1* mRNA is post-transcriptionally regulated to prevent EGL-1 from being produced in cells destined to live, or because small levels of EGL-1 might be below a threshold required to induce cell death. Such a threshold might be determined by the level of CED-9, CED-4, and CED-3 in a cell, and this threshold might vary

between cells. As this study only examined mRNA transcripts, the level of cell-death proteins is still unexamined.

A common control for smFISH experiments to ensure that signal is not due to nonspecific binding is to perform smFISH against a gene containing a large deletion (Ji and van Oudenaarden, 2012). I performed smFISH in *ced-3(n3692)* mutant males, which contain a large deletion spanning -410 bp to +240 bp relative to the start codon of *ced-3.* This mutation eliminates part of the promoter, the first exon, and part of the second exon of *ced-3*. Although this deletion removes parts of the promoter and the gene, *ced-3* smFISH probes bound to transcripts in similar tissues in *ced-3* mutants as in wild-type animals (*e.g.* male tail, linker cell, and germline). Daniel Denning similarly detected transcripts of *ced-3* in this deletion mutant by RT-PCR (personal communication). Thus, this deletion does not prevent the transcription of *ced-3*, and the presence of *ced-3* transcripts detected by smFISH does not necessarily represent nonspecific binding to other mRNA transcripts.

Although deletion strains suitable for use as negative controls are not available for the cell death genes, other lines of evidence suggest that the pattern of transcripts I have detected by smFISH are likely to represent the endogenous cell death gene patterns rather than nonspecific binding. As discussed above, *egl-1* transcripts were mostly detected at times and in cells that were in positions consistent with the initiation of cell death. In the late male tail, where I am able to more accurately determine cell identities, *egl-1* was almost exclusively found in dying cells, and expression in living cells was limited to a very small number of transcripts. This is a subset of the pattern of *egl-1* gene expression as assayed by P_{egl-}

1::4xNLS::gfp expression (*e.g.* see Chapter 2, Figure 2A), suggesting that the *egl-1* smFISH is unlikely to be binding to targets other than *egl-1*. I detected transcripts of *ced-4* and *ced-3* in the germline of males and hermaphrodite animals. These proteins have been detected in male and hermaphrodite germlines by antibody staining (Jaramillo-Lambert *et al*., 2010; Silva *et al*., 2013). I detected transcripts of *ced-9* broadly throughout the worm; this is consistent with the finding that *ced-9* is required in many cells to prevent cell death (Hengartner *et al*., 1992). I detected high levels of *ced-3* transcripts in the dying linker cell, which was surprising because the linker cell death is caspase independent (Denning *et al*., 2013). However, a role for *ced-3* in the linker cell corpse degradation has more recently been observed (Lena Kutsher, personal communication; see Chapter 5), suggesting that *ced-3* is likely expressed in the dying linker cell. I detected *ced-3* transcripts in other cells of the fourth-larval stage male tail; *gfp* driven by the *C. briggsae ced-3* promoter is also expressed in the fourth-larval stage male tail (Maurer *et al.,* 2007). GFP constructs driven by cell death genes can interfere with the endogenous cell death program (Maurer *et al.,* 2007), but smFISH is a technique that is powerful and does not disrupt endogenous gene activity.

Materials and Methods

Strains and Genetics

C. elegans was maintained on nematode growth medium (NGM) Petri plates at 20°C (Brenner, 1974). Animals analyzed in the first, second, and third larval stages were of the genotype *enIs64; unc-76.* Animals analyzed in the fourth larval stage were of the genotype *nIs343; him-5(e1490); nIs349*. Mutations of *him-8* or *him-5* cause a high incidence of males among progeny (Hodgkin, 1983). The following mutations, integrations, and extrachromosomal arrays were used: LGI: *enIs64*[P_{ced-1}*::gfp::rab-7, unc-76 (+)*]. LGII: *nIs343* [P_{egl-1}*::4xNLS::gfp, lin-15AB(+)*]. LGV: *him-5(e1490), unc-76(e911)*. LGX: *nIs349* [P_{ceh-28}*::mCherry, lin-15AB(+)*].

Single-Molecule Fluorescent *in Situ* Hybridization

To synchronize worms, gravid animals were bleached in 4.4% NaHOCl with 0.7 M NaOH for 10 minutes with periodic vortexing to recover eggs. These eggs were hatched in M9 solution without food at 20°C overnight or for a full day at 15°C to synchronize worms in the first larval stage. The hatched worms (and some remaining unhatched eggs) were recovered on NGM Petri plates with cultures of OP50 *E. coli* bacteria and grown at 20°C until they reached the desired age for fixation.

Fixation of larval animals, conjugation of fluorescent probes to and purification of oligo probes, hybridization and imaging were performed as previously described (Denning *et al.,* 2013), using 4% paraformaldehyde in nuclease-free phosphate buffered saline (Ambion) as a fixation solution. For analysis of first, second, and third larval stage animals, the *egl-1* set of probes included 24 20-nucleotide probes complementary to regions in all exons and

untranslated regions of *egl-1*. To avoid cross-reactivity with *nIs343* transcript, for analysis of fourth larval stage animals the *egl-1* set of probes contained only a subset of 21 of these probes, complementary to regions in the second and third exons and 3' untranslated region of *egl-1.* The *egl-1* probe sets were each conjugated to the fluorophore Alexa 594 (Invitrogen). The *ced-3* set of probes included 48 20-nucleotide probes complementary to regions in all exons of *ced-3*. The *ced-3* probe set was conjugated to Cy5 (Invitrogen). The *ced-4* set of probes included 48 20-nucleotide probes complementary to all exons of the short pro-apoptotic isoform of *ced-4* conjugated to Alexa 594. The *ced-9* set of probes included 48 20-nucleotide probes complementary to all exons and 3' UTR of *ced-9* conjugated to Cy5. Image processing was done using Fiji. Empirically, the Fiji processing tool "Find Edges" was found to eliminate much of the background while highlighting most of the transcripts and so was used for background subtraction.

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Figure 1: Timings of somatic cell divisions and cell deaths in *C. elegans* during larval development. Blue diamonds mark the occurrence of a single cell division. No. cells refers to the number of cells ever generated the larval worm, *i.e.* 1 + 2 x the number of divisions that have occurred. Therefore, a horizontal series of blue diamonds (*e.g.* at 17 hours) implies a number of cell divisions that occur at the same time, while a vertical gap between diamonds (*e.g.* at 370 cells) implies a quiescent period of no somatic cell division. Colored squares mark the timing of developmental events: L1, first larval stage; L2, second larval stage; L3, third larval stage. Black circles mark the timing of a cell death. Translucent blue rectangles mark the approximate timing of fixations. Data is courtesy of N. Bhatla, based on Sulston and Horvitz, 1977 and Sulston *et al.,* 1983.

Figure 1

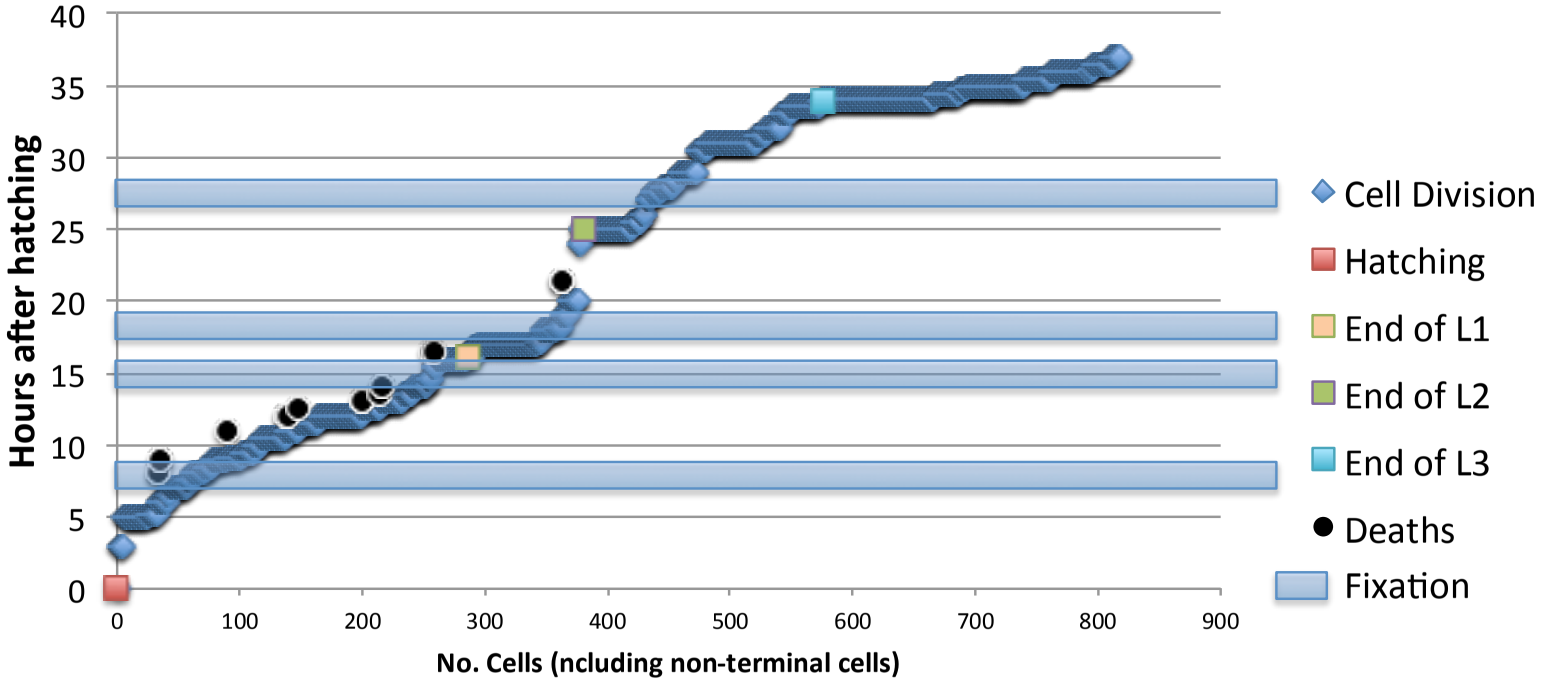

Hours after hatching
40
35
30
25
20
15
10
5
0
0
100
200
300
400
500
600
700
800
900
No. Cells (ncluding non-terminal cells)
Cell Division
Hatching
End of L1
End of L2
End of L3
Deaths
Fixation

Figure 2: Expression of cell death genes in animals aged for 8.3 hours. **a,b** *ced-3* (red) and *ced-4* (yellow) mRNA is sparsely located throughout the body in two representative animals. *ced-3* mRNA seem to be most concentrated in the germline (gl). **c,d** *ced-9* (red) mRNA is sparsely located throughout the body in two representative animals. *egl-1* mRNA seem to be located in only a few cells (*e.g.* white arrowhead). Scale bars: 10 μm.

Figure 2

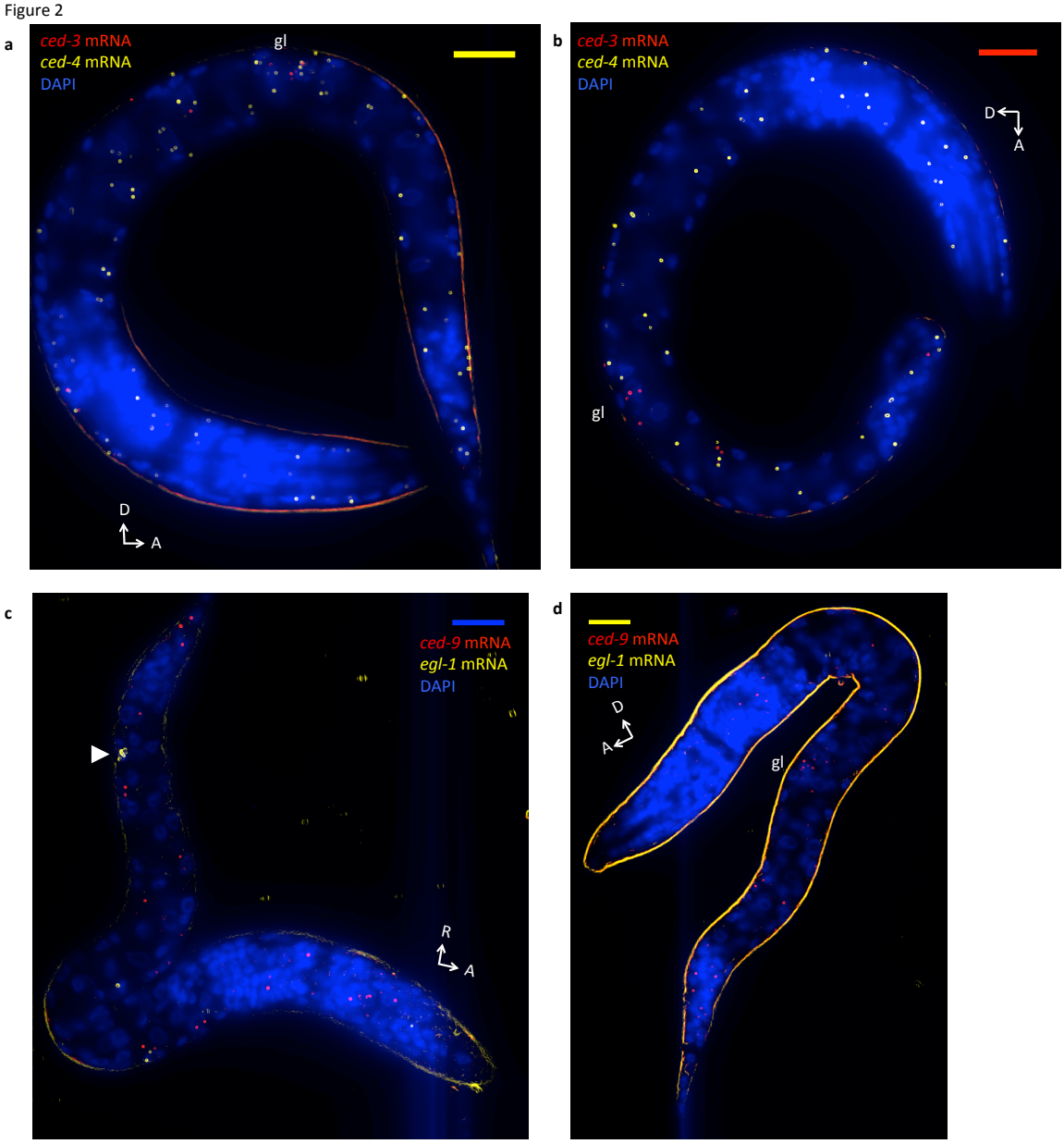
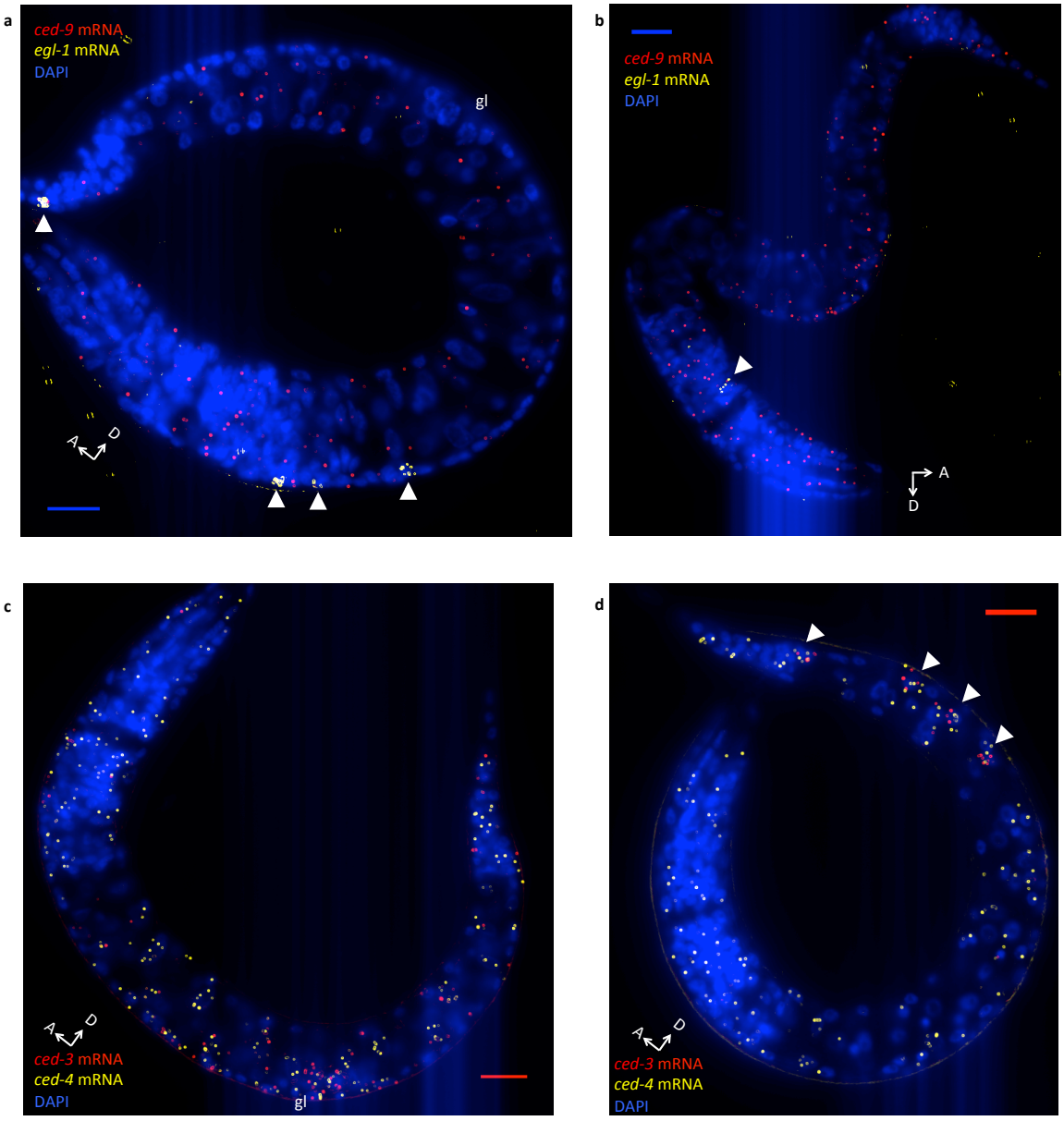

a
ced-3 mRNA
ced-4 mRNA
DAPI
gl
D
A
b
ced-3 mRNA
ced-4 mRNA
DAPI
D
A
gl
c
ced-9 mRNA
egl-1 mRNA
DAPI
R
A
d
ced-9 mRNA
egl-1 mRNA
DAPI
D
A
gl

Figure 3: Expression of cell death genes in animals aged for 15.5 hours. **a,b** *ced-9* (red) mRNA is sparsely located throughout the body in two representative animals. *egl-1* mRNA seem to be located in only a few cells (*e.g.* white arrowheads). **c,d** *ced-3* (red) and *ced-4* (yellow) mRNA is sparsely located throughout the body in two representative animals. **c**, *ced-3* mRNA seems to be especially concentrated in the germline (gl). **d**, *ced-3* mRNA is concentrated in four cells in the posterior ventral cord (white arrowheads). Scale bars: 10 μm.

Figure 3


a
ced-9 mRNA
egl-1 mRNA
DAPI
gl
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egl-1 mRNA
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ced-3 mRNA
ced-4 mRNA
DAPI
gl
d
A
D
ced-3 mRNA
ced-4 mRNA
DAPI

Figure 4: Expression of cell death genes in animals aged for 18 hours. **a,b** *ced-9* (red) mRNA is sparsely located throughout the body in three representative animals. *egl-1* mRNA seems to be located in only a few cells (white arrowheads) in the head or tail (**a**) or in the posterior ventral cord, outlined in white dashed lines (**b**). **c,d** *ced-3* (red) and *ced-4* (yellow) mRNA is sparsely located throughout the body in two representative animals. **d,** *ced-3* mRNA seems to be especially concentrated in the germline (gl). Scale bars: 10 μm.

Figure 4

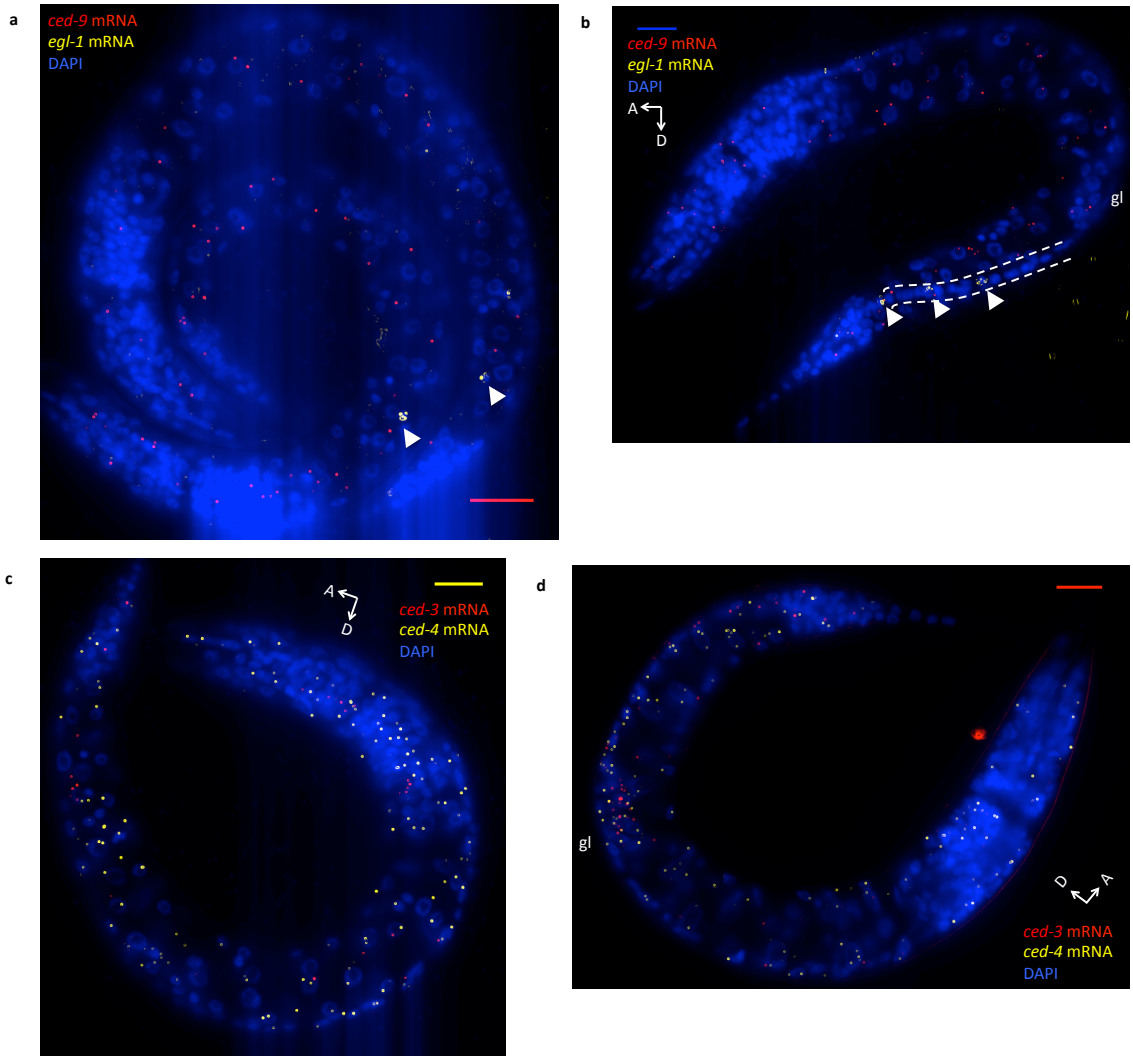

a
ced-9 mRNA
egl-1 mRNA
DAPI
b
ced-9 mRNA
egl-1 mRNA
DAPI
A
D
gl
c
A
D
ced-3 mRNA
ced-4 mRNA
DAPI
d
gl
D
A
ced-3 mRNA
ced-4 mRNA
DAPI

Figure 5: Expression of cell death genes in animals aged for 28 hours. **a,c** *ced-3* (red) and *ced-4* (yellow) mRNA is sparsely located throughout the body in two representative animals. *ced-3* mRNA seem to be most concentrated in the germline (gl). **b,** *ced-3* is highly expressed in one cell that might be a dying cell in the posteirid (white arrowhead). This image is from the same animal as in panel a, but is in a slightly different focal plane **d-g** *ced-9* (red) mRNA is sparsely located throughout the body in two representative animals. *egl-1* mRNA seem to be located in only a few cells (white arrowheads) in the head (**e**) or what appears to be the postdeirid (**f**). Panels d and e are images from the same animal but are from different focal planes. Panels f and g are images from the same animal but are from different focal planes. Scale bars: 10 μm.

Figure 5

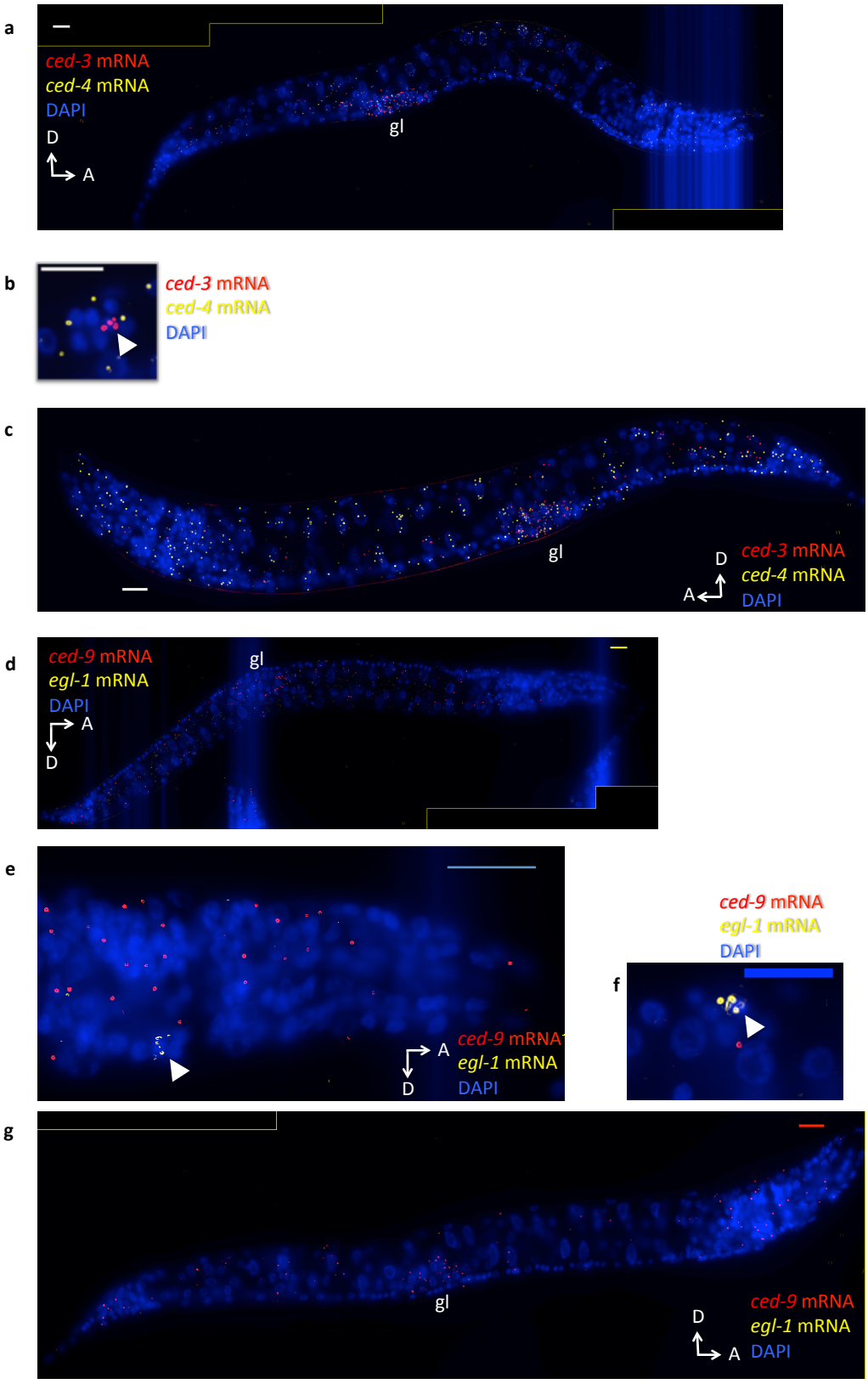

a
ced-3 mRNA
ced-4 mRNA
DAPI
D
A
gl
b
ced-3 mRNA
ced-4 mRNA
DAPI
c
gl
D
A
ced-3 mRNA
ced-4 mRNA
DAPI
d
ced-9 mRNA
egl-1 mRNA
DAPI
A
D
gl
e
A
D
ced-9 mRNA
egl-1 mRNA
DAPI
f
ced-9 mRNA
egl-1 mRNA
DAPI
g
gl
D
A
ced-9 mRNA
egl-1 mRNA
DAPI

Figure 6: Expression of cell death genes in the developing male tail. **a,b** *ced-3* (red) and *egl-1* (green) in the tails of two representative animals approximately 34-35 hours old. **a**, *ced-3* mRNA seem expressed throughout the ray sublineages, but *egl-1* mRNA is restricted to a small number of cells (white arrowheads). Based on position, these are likely dying Rn.aap cells. **b,** *egl-1* is expressed in a small number of cells in positions consistent with cells fated to die: B.al/rapaav (1), B.ppaa (2); and B.γal/rd, B.alappd, and/or B.arappd (3). *ced-3* is expressed in B.al/rapaav (1) and B.γal/rd, B.alappd, and/or B.arappd (3). Scale bars: 10 μm.

Figure 6

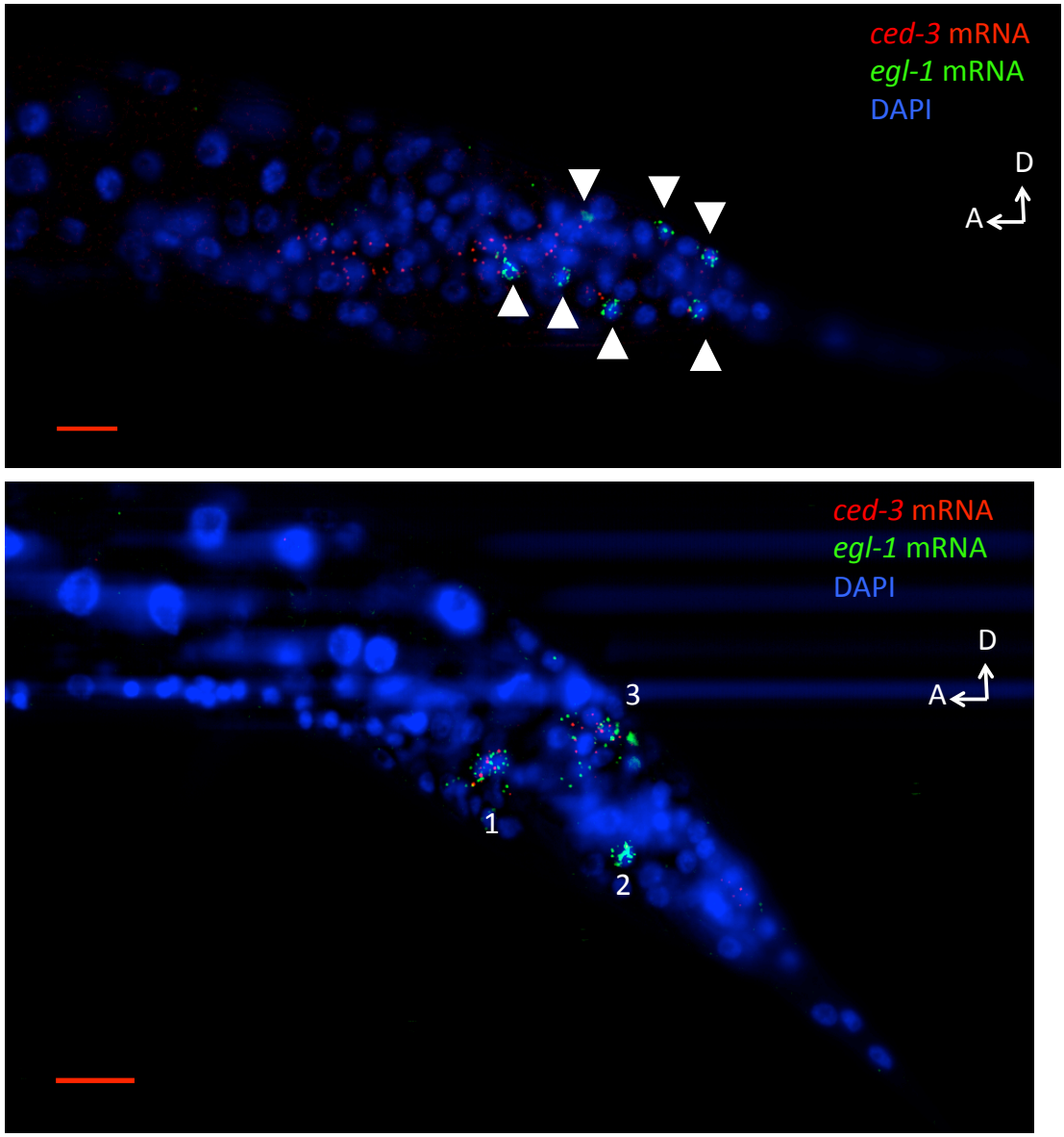
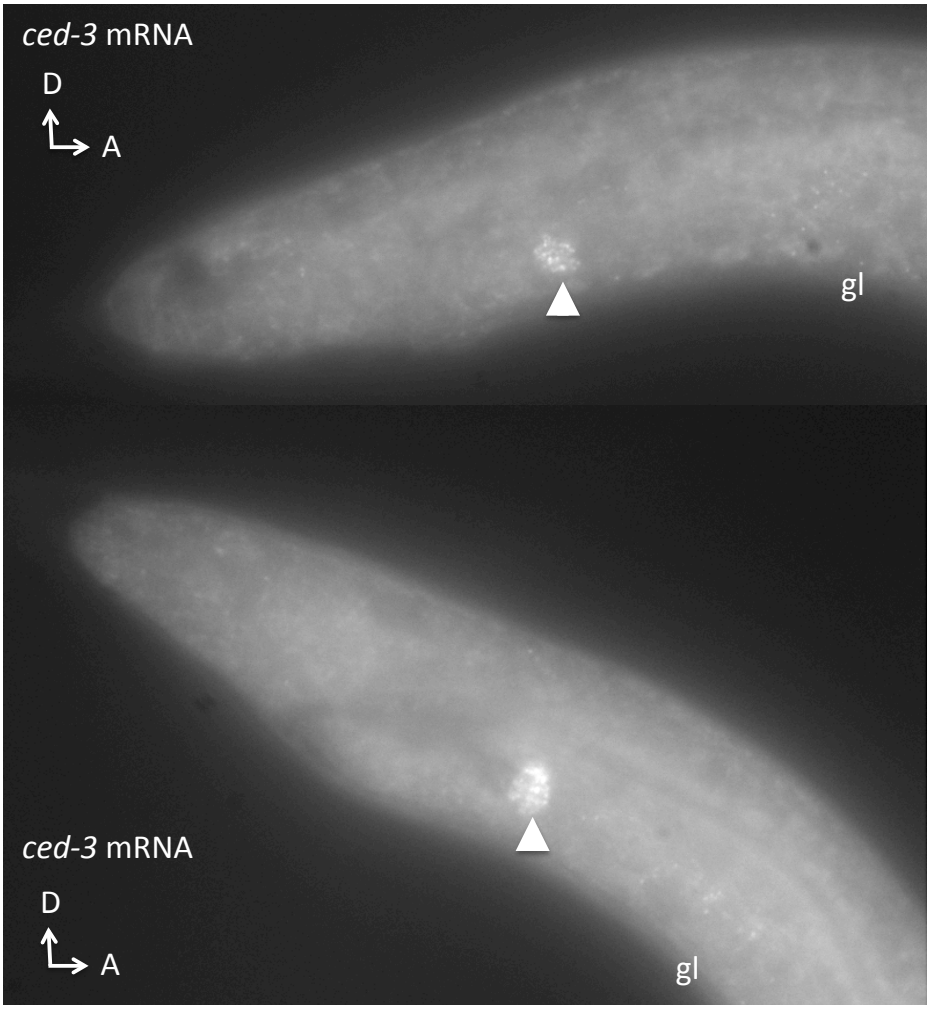


Figure 7: Expression of *ced-3* in the linker cell. **a,b** *ced-3* (white) is expressed in two representative animals in the late fourth larval stage. Based on the shape of the tail, these animals are approximately 41-44 hours old. *ced-3* transcripts are also visible in the male germline (gl).

Figure 7



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Chapter 5

Future Directions

Assisted Suicide

In this thesis, I have established that certain cells in *C. elegans* die by a novel mechanism, assisted suicide. I chose the cells B.alapaav and B.arapaav for study based on the observation that ablation of the engulfing cell, P12.pa, is able to prevent the death of B.al/rapaav (Sulston *et al.*, 1980). However, this finding was incomplete; I found that removing P12.pa can prevent the death, but does not always (B.al/rapaav died in 9 of 23 animals following P12.pa ablation). It is likely that Sulston's reports were based on a small number of laser ablation experiments. Furthermore, I have shown that even if the normal engulfing cell is absent, a cell can be engulfed by a different neighboring cell. These findings demonstrate that laser ablation of engulfing cells is an incomplete way to search for cases of cellular assisted suicide. Thus, it is possible that there could be other cases of assisted suicide in *C. elegans*.

While observing the B.al/rapaav death in a variety of mutant backgrounds, I observed other cells in the developing male tail that appeared to behave similarly to B.al/rapaav. Specifically, in engulfment mutants, there are cells that adopt a persistent cytoplasmic refractility but never take on the fully-refractile morphology typical of corpses. These cells are presumably Bγ.al/rd, B.alappd, and/or B.arappd, since these are the only cell deaths expected in this time and place. All three deaths are unlikely to be engulfment-dependent, since I have observed worms with no more than two persistent corpses in this region. It is possible that one of these cell deaths is completely engulfment dependent or that more than one of these cell deaths are strongly promoted by engulfment.

Bγ.ald and Bγ.ard share many features with B.alapaav and B.arapaav. Both pairs of cells form an equivalence group, in which one cell dies and one cell survives (Sulston *et al.*, 1980). Each of these cells is generated near the end of the third larval stage, and the cells that die do so approximately five or more hours after their generation. Five hours is an exceptionally long life for a cell that is fated to die in *C. elegans*. Based on these similarities, combined with the observations of apparently undead cells in engulfment mutants, I suspect that the Bγ.al/rd death might also represent an assisted suicide. Sulston reported that ablation of the Bγ.al/rd engulfing cell, the F.l/rd syncytium, did not prevent the Bγ.al/rd death (Sulston *et al.*, 1980). If the Bγ.al/rd death is an assisted suicide, it might be particularly accessible to other neighboring cells that could engulf it in F.l/rd's absence.

To demonstrate that Bγ.al/rd is an assisted suicide, it would need to be determined that the Bγ.al/rd death is both engulfment- and caspase-dependent, but that the initiation of the cell death process is not engulfment-dependent, similar to the experiments that have been described in this thesis concerning the B.al/rapaav death.

Regulation of the B.al/rapaav equivalence group cell-fate decision

My studies of the B.al/rapaav cell death were primarily focused on the execution phase of the cell death process. However, the question of how the decision of which cell adopts which fate is also likely to be a line of research with opportunities for rich discovery. My preliminary observations suggest that the *lin-*

12 signaling might not be responsible for mediating the left-right, death-survival fate decisions, as I observed one (never two) corpses in a small number of *lin-12(gf)* and *lin-12(lf)* mutants (*n* = 2-3). However, this should be more rigorously tested, along with mutants in other candidate pathways, such as Wnt, EGF, and FGF. Care must be taken to observe the cell lineage in full, as the development of the male tail and B cell lineage require many signaling pathways, and it is likely that in these mutants B.alapaav and B.arapaav might be generated or located abnormally (Chamberlin and Sternberg, 1993, 1994). For this reason, it is also possible that mutants in signaling pathways might non-specifically affect the death-survival fate decisions of the B.al/rapaav equivalence group. For example, if B.alapaav and B.arapaav detect each other by touching to make a death decision, they might both survive if they are not located next to one another in some mutant background, even if that mutation is not in the gene required for their communication. On the other hand, if a protective signal at the midline prevents the death of only one of B.alapaav and B.arapaav, they might both die if extra cells are generated that prevent their migration to the midline.

An unbiased way to identify factors involved in the regulation of the fate decision in the B.al/rapaav equivalence group would be to conduct a genetic screen. As described in this thesis, $P_{egl\text{-}1}$*::gfp* is expressed strongly in the secondary B.al/rapaav that is fated to die, and weakly if at all in the primary B.al/rapaav homolog that is fated to survive. To find mutations that cause both cells to adopt the secondary fate, one could mutagenize animals of a $P_{egl\text{-}1}$*::gfp; ced-3 him-8* background and isolate mutants with two strongly GFP-expressing cells in the

proctodeum. To find mutations that cause both cells to adopt the primary fate, one could isolate mutants with little-to-no GFP expression in the proctodeum. The latter class of mutants would likely include many nonspecific mutations that prevent the formation of B.al/rapaav or cause transgene silencing. Since the $P_{egl\text{-}1}$*::gfp* is expressed in cells other than B.al/rapaav, it might be desirable to try variations on the *egl-1* promoter region used to identify a construct with a more restricted expression pattern. Alternatively, it might be possible to use other types of reagents, such as split GFP, to limit the expression pattern to just B.alapaav and B.arapaav. This technique, also referred to as bimolecular fluorescence complementation, entails expressing two individually non-fluorescent peptides from two different promoters (Hu *et al.,* 2002). Only in cells that express both proteins will the two peptides interact to create a fluorescent dimer. Therefore, using two different promoters that drive expression in two different sets of cells, one can create a reporter for the overlap between the two sets of cells.

Regulation of cell-death genes

While conducting single-molecule FISH experiments to determine expression patterns of cell death genes, I observed that transcriptional regulation of *ced-3* might be more cell-specific than has previously been appreciated. Little has been published about the pattern of expression of cell-death genes; cell-specific upregulation of *ced-3* has previously been characterized only for the hermaphrodite CEM and tail-spike cell deaths (Maurer *et al.,* 2007; Nehme *et al.*, 2010). These reports were based on expression of multicopy transgene constructs, not on

endogenous mRNA analysis or by tagging the endogenous gene. My preliminary observations indicate that *ced-3* transcription might occur in specific cells of the developing male tail, probably Bγ.ald or Bγ.ard, B.alappd, and B.arappd. In addition, *ced-3* appears to be expressed broadly in the cells of the ray sublineages, including both the dying Rn.aaps and other surviving cells of the rays. It is possible that *ced-3* is generally transcribed in nascent cells following cell division, but that a second wave of *ced-3* transcription is required for the cell death of "old" cells, after the previous stores of CED-3 have turned over.

In the case of the tail-spike cells, *ced-3* upregulation is sufficient to promote cell death even without the activity of *egl-1* (Maurer *et al.*, 2007). However, *ced-3* is not sufficient for the B.al/rapaav cell death; *egl-1* is also required (see Chapter 2, Figure 1D). Quantitative approaches to measure the cell-death gene transcript levels by smFISH and protein levels by tagging each gene with fluorescent proteins or tags that facilitate antibody staining (*e.g.* using CRISPR-Cas9) could reveal important insights into how living and dying cells express the cell death genes. For example, are there differences in expression levels of pro- and anti-apoptotic genes (or isoforms) between living and dying cells such that they can be clustered into two discrete classes, or do all cells exist along a range of sensitivity to cell death based on their cell-death gene expression levels? Would these expression profiles give insight into why some cells are harder to kill than others? The answer to this question will be informative to the area of cancer and cancer therapeutics, since treatments of tumors are only useful if there is a therapeutic window in which treatment induces cell death of cancer cells but not of normal cells. Additionally, this

information could be used to design more efficient genetic screens for the study of cell death. Do all dying cells express *egl-1*, and do all living cells repress *egl-1*?

There is already precedence for unexpected gene expression patterns to lead to new discoveries. I found that the dying linker cell often contained large numbers of *ced-3* mRNA transcripts, even though this cell death is considered to be caspase-independent (Chapter 4, Figure 7). This finding prompted Lena Kutsher in the Shaham Lab to re-examine the linker cell death in the *ced-3* mutant. She discovered while unnecessary for the linker cell death, *ced-3* promotes the efficient degradation of the linker cell corpse (personal communication). Thus, while the linker cell death is caspase-independent, the dying cell apparently produces capsases to promote its own degradation. In this way, knowledge of gene expression patterns at the individual cellular level can inspire experimentation that has potential to reveal new insights into cell and developmental biology. Therefore, I expect that further probing of cell-death gene expression levels in a cell-type-specific manner will be highly informative.

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