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Genetic and Molecular Characterization of Programmed Cell Death in the C.elegans Tail-Spike Cell

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GENETIC AND MOLECULAR CHARACTERIZATION OF PROGRAMMED CELL DEATH IN THE *C. ELEGANS* TAIL-SPIKE CELL

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Work in *Caenorhabditis elegans* has been instrumental in deciphering the molecular basis of programmed cell death. However, despite extensive characterization of broad-acting cell death genes, the molecular events triggering cell-specific activation of the cell death machinery remain, for the most part, unknown. In some *C. elegans* somatic cells, transcription of the *egl-1*/BH3-only gene is believed to promoted cell-specific death. EGL-1 protein inhibits the CED-9/Bcl-2 protein, resulting in release of the caspase activator CED-4/Apaf-1. Subsequent activation of CED-3 caspase by CED-4 leads to cell death. But despite the important role of *egl-1* transcription in promoting CED-3 activity in cells destined to die, it remains unclear whether temporal control of cell death is mediated by *egl-1* expression.

Here, we establish the *C. elegans* tail-spike cell as an attractive model for studying the initiation of programmed cell death. We show that, while death of the tail-spike cell is dependent upon the *ced-3* and *ced-4* genes, *egl-1* and *ced-9* play only a minor role in the death of this cell, demonstrating that temporal control of cell death can be achieved in the absence of *egl-1*. We go on to show that the timing of tail-spike cell death onset is controlled by transcriptional induction of the *ced-3* caspase. In the tail-spike cell, *ced-3* expression is induced minutes before the cell dies, and this induction

is sufficient to promote the cell's demise. Both *ced-3* expression and cell death are dependent upon the transcription factor-encoding gene *pal-1*, the *C. elegans* homolog of the mammalian tumor suppressor gene Cdx2. PAL-1 can bind to *ced-3* promoter sites critical for tail-spike cell death, suggesting that it promotes cell death by directly activating *ced-3* transcription. Our results highlight a previously undescribed role for transcriptional regulation of caspases in controlling the timing of cell death onset during animal development.

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Chapter 1

Transcriptional mechanisms governing the specification of programmed cell death

Programmed cell death (PCD) has long been recognized as playing a critical role during organismal development. In the middle of the 19th century, the German pathologist Rudolph Virchow described a type of cell death that he pronounced to be "natural as opposed to violent"; he termed this process necrobiosis, acknowledging the ying-yang relationship between cellular destruction and organismal survival (Virchow, 1860). The significance of programmed cell death was further elaborated upon in the middle of the 20th century, when Glucksmann articulated its role in embryonic development, metamorphosis and the destruction of vestigial tissues (Glucksmann, 1951; Glucksmann, 1965). Around this time, the embryologist Saunders observed that cell death in chick embryos took place in a reproducible pattern (Saunders, 1966), suggesting that cell death was in fact carefully regulated. In their seminal and often cited paper, Kerr, Wyllie and Currie put forth the idea that these naturally occuring programmed cell deaths follow a distinct pattern of morphological changes. They suggested that programmed cell death was a distinct cellular fate, just like cell division, migration or differentiation, and coined this fate "apoptosis", deriving the word from the Greek for "falling off" (1972).

This fate is adopted by a vast number of cells, in nearly all metazoans. Programmed cell death is used to destroy cells that have been produced in excess and

to ablate cells that have become superfluous, ultimately serving to shape an intricate body plan. The importance of PCD to the developing organism is highlighted by the phenotypes of animals with cell death defects. Mice with targeted gene disruption of either caspase-9 or caspase-3/CPP-32 – critical cell death effectors – die perinatally with severe brain abnormalities resulting from reduced apoptosis (Kuida et al., 1998; Kuida et al., 1996). Non-lethal defects in components of the cell death machinery have been implicated in a variety of diseases, including cancer, autoimmune syndromes and neurodegenerative disorders. Soengas and colleagues have reported that a significant fraction of cell lines derived from metastatic melanomas express low or undetectable levels of the cell death activator Apaf-1. In addition to contributing to cancer progression, decreased Apaf-1 expression also resulted in resistance to chemotherapy-induced cell death (Soengas et al., 2001). Mutations in the cell death executioner caspase-8 have been correlated to an increase in neuroblastoma metastasis (Stupack et al., 2006), as well as to the progression of colorectal tumors from non-malignant adenomas to invasive carcimonas (Kim et al., 2003). Abnormalities in caspase-10 have been implicated in both gastric cancer (Park et al., 2002) as well as the debilitating autoimmune lymphoproliferative syndrome type II (Wang et al., 1999). In some instances, activation of the cell death machinery can also contribute to disease. In mouse stroke models, caspase-3 is activated in neurons undergoing ischemic cell death (Namura et al., 1998); caspase-3-mediated apoptosis also takes place in lymphocytes as a counterintuitive response to bacterial sepsis (Hotchkiss et al., 2000). In both of these cases, caspase inhibitors have been demonstrated to have a therapeutic effect, resulting in neuronal protection (Hara et al., 1997) as well as enhanced immunity (Hotchkiss et al., 2000).

C. elegans as a model for studying the initiation of programmed cell death

With its sequenced genome, large brood size, short lifespan and self-fertilizing mode of reproduction, *C. elegans* is a powerful system for forward genetics; the nematode's fixed lineage also makes it an ideal model organism for studying the determination of developmental cell fate. In particular, work in *Caenorhabditis elegans* has been instrumental in deciphering the molecular basis of programmed cell death. In every wild-type *C. elegans* hermaphrodite, 1090 cells are born, 131 of which die (Sulston and Horvitz, 1977; Sulston et al., 1983). The exact location and time of these deaths is known, thanks to the heroic efforts of researchers who followed every cell in the worm throughout its development, noting the time of every cell birth, every cell division and every cell death (Sulston and Horvitz, 1977; Sulston et al., 1983). The fixed pattern of cell death in *C. elegans* allows the unambiguous identification of dying cells, which can be visualized using differential interference contrast (DIC) optics by their distinct "button-like" morphology. Importantly, "undead" cells, those destined to die but whose death has been inappropriately blocked, can also be readily visualized and identified. A genetic pathway governing the execution of cell death was first identified in *C. elegans*, and the machinery responsible for executing cell death in this animal is conserved in many metazoans (reviewed in Metzstein et al., 1998).

The downstream-most player in the *C. elegans* execution pathway is *ced-3*, a member of the caspase family of proteases. Three genes act upstream of *ced-3* in this pathway: *ced-4*/Apaf-1, *ced-9*/Bcl-2 and *egl-1*/BH3-only. Loss-of-function mutations in *ced-3*, *ced-4* and *egl-1* block programmed cell death (Ellis and Horvitz, 1986; Conradt and Horvitz, 1998), indicating that these genes possess pro-apoptotic activity. Conversely, *ced-9* loss-of-function mutations result in lethality as a result of extensive ectopic cell death (Hengartner et al., 1992), indicating that *ced-9* possesses anti-apoptotic activity. Consistent with this conclusion, a *ced-9* gain-of-function (gf)

mutation blocks cell death (Hengartner and Horvitz, 1994). Epistasis experiments have placed these genes into a linear pathway, with *egl-1*, *ced-9*, *ced-4* and *ced-3* acting in that order to direct cellular demise (reviewed in Metzstein et al., 1998).

Genetic, biochemical and structural studies have shed light on the complex interplay between these four genes. CED-3 activation is dependent upon its autocatalytic cleavage and assembly into a heterodimer composed of two "small" and two "large" CED-3 subunits (reviewed in Yan and Shi, 2005). Proteolytic activation of CED-3 is facilitated by its interaction with the tetrameric CED-4 adaptor protein (Yang et al., 1998; Yan et al., 2005). In most *C. elegans* cells, CED-4 is sequestered at the mitochondria as a result of its interaction with CED-9 (Chen et al., 2000). This interaction maintains CED-4 in a dimeric, inactive state (Yan et al., 2005), blocking CED-4 mediated activation of CED-3 and preventing cell death (Chen et al., 2000; Chinnaiyan et al., 1997). EGL-1 binding to CED-9 disrupts the interaction between CED-4 and CED-9 (del Peso et al., 1998; Yan et al., 2004), resulting in the release of CED-4 from the mitochondria, and thereby promoting CED-3 activation and cellular demise (Chen et al., 2000).

Mutations in *ced-3*, *ced-4*, *ced-9* and *egl-1* affect most somatic cells in *C. elegans*, indicating that these genes function as part of a general cell death pathway. Genes involved in the engulfment and nuclear degradation of dying cells in *C. elegans* have also been identified and characterized, and, similarly, these genes exert their influence on most dying cells (Ellis and Horvitz, 1991; Stanfield and Horvitz, 2000; Hedgecock et al., 1983; Wu et al., 2001; Parrish et al., 2001; Wang et al., 2002; Wu et al., 2000).

Despite extensive characterization of broad-acting cell death genes, the molecular events triggering cell-specific activation of the cell death machinery remain, for the most part, unknown. Work in *C. elegans* suggests that members of the "core" cell death execution machinery – *ced-3*, *ced-4* and *ced-9* – are constitutively expressed in most cells at levels sufficient to execute a cell's death program (Shaham and Horvitz,

1996b). However, only 11% of *C. elegans* cells are fated to die (Sulston and Horvitz, 1977; Sulston et al., 1983). What triggers death in these cells? Progress towards answering this question has been hindered by the difficulty of designing genetic screens geared to detect cell-specific cell death defects. Some of the initial cell death screens identified animals defective in cell death execution by their absence of cell corpses (Ellis and Horvitz, 1986); animals with mutations in the engulfment machinery were identified by their excess of these corpses (Ellis et al., 1991). Similar approaches would be very difficult in large-scale screens aimed at isolating mutations affecting only a few cell deaths. Instead, screens designed to isolate cell-specific regulators of cell death have relied upon cell-specific markers. The scarcity of these markers has hampered progress, and the mechanisms underlying cell death specification have been identified in only 7 of the 131 dying cells in *C. elegans*.

How does a given cell know that it is fated to die? In a developing organism, timing is also critical – how does a cell know *when* it is supposed to die? How does it initiate cell death? This chapter will discuss the mechanisms currently proposed to regulate cell death specification and cell death initiation in *C. elegans*. Intriguingly, cell death specification in *C. elegans* appears to be, for the most part, regulated transcriptionally. Studies in *Drosophila* and various mammalian systems have also advanced our understanding of cell death specification. This chapter will describe some of the contributions from these systems, focusing upon transcriptional mechanisms of cell death specification.

Inappropriate activation of *egl-1* expression can promote ectopic cell death

In a subset of somatic cells in *C. elegans*, the *egl-1* gene is believed to be a critical mediator of a cell's decision between life and death. Transcription of *egl-1* is induced in some cells destined to die, leading to the hypothesis that such transcription may determine the timing of cell death initiation. Several regulators of *egl-1* transcription

have been identified, and mutation of these regulators or alteration of their binding sites in the *egl-1* promoter can inhibit or promote cell-specific cell death.

egl-1 was originally isolated in a screen for animals with an egg-laying defective (*egl*) phenotype (Trent et al., 1983). In animals carrying an *egl-1* gain-of-function mutation, the two hermaphrodite-specific neurons (HSNs), which are normally required for egg-laying, die inappropriately. As a result, eggs are not laid, and instead accumulate within the hermaphrodite gonad. Several *egl-1*(gf) alleles have been isolated. Each of these alleles contains a single base pair (bp) mutation 5.6 kb downstream of the start of the *egl-1* transcription unit, within a region containing a putative TRA-1A binding site (Conradt and Horvitz, 1999). TRA-1A is a transcriptional regulator of sexual dimorphism in *C. elegans*; strong loss-of-function mutations in the *tra-1* gene result in almost complete transformation of hermaphrodites into males (Hodgkin, 1987; Zarkower and Hodgkin, 1992). Conradt and Horvitz (1999) have shown that TRA-1A is able to bind to the putative TRA-1A binding site within the *egl-1* regulatory region, and that mutations similar to those found in *egl-1*(gf) mutant animals disrupt this interaction. They suggest that *tra-1* normally represses *egl-1* expression in the HSNs, thereby blocking cell death. Mutation of the TRA-1A binding site results in ectopic *egl-1* expression, and subsequent death of the HSNs. Consistently, *tra-1*(gf) mutations, in which *tra-1* is overexpressed in males, block the death of the male HSNs; *egl-1*(gf) mutations suppress this inappropriate survival (Conradt and Horvitz, 1999).

ces-1 and *ces-2* act in a transcriptional cascade upstream of *egl-1*

Two additional regulators of cell death, *ces-1* and *ces-2*, were originally isolated in a screen for mutants with abnormal patterns of serotonin expression (Trent, 1982). While the *C. elegans* pharynx usually contains two serotonergic cells, the bilaterally symmetrical NSMs (neurosecretory motor neurons), Trent found that *ces-1*(gf) and

ces-2(lf) mutant animals contained four cells staining with an anti-serotonin antiserum. The extra two serotonergic cells were identified as the sister cells of the NSMs (Ellis and Horvitz, 1991), cells that normally die during early embryogenesis (Sulston et al., 1983). In addition to blocking the death of the NSM sister cells, the *ces-1*(gf) mutation also blocked the death of the I2 sister cells. Other cell deaths in the worm appeared to be unaffected by *ces-1* and *ces-2* mutations (Ellis and Horvitz, 1991), suggesting that *ces-1* and *ces-2* might regulate cell-specific cell death.

Subsequent genetic and biochemical studies have placed *ces-1* and *ces-2* into a transcriptional cascade believed to regulate the expression of *egl-1*. Both genes encode transcription factors: CES-1 is a member of the Snail family of zinc finger proteins (Metzstein and Horvitz, 1999), and CES-2 belongs to the bZIP subfamily of transcription factors (Metzstein et al., 1996). The cell death defect of *ces-2*(lf) mutant animals is suppressed by *ces-1*(lf) mutations (Ellis and Horvitz, 1991), indicating that pro-apoptotic *ces-2* may act upstream of anti-apoptotic *ces-1*. Indeed, CES-2 is able to bind a region of the *ces-1* promoter containing a *ces-2* consensus DNA binding site (Metzstein and Horvitz, 1999), suggesting that *ces-2* may directly inhibit the expression of *ces-1*. It has been speculated that *ces-1* may act in turn by directly inhibiting *egl-1* expression. Notably, recent studies have shown that a *ces-1*(gf) mutation blocks expression of an *egl-1* transcriptional reporter (Thellmann et al., 2003). In addition, CES-1 is capable of binding a conserved region of the *egl-1* promoter demonstrated to be required for *egl-1* expression in the NSM sister cells (Thellmann et al., 2003). The *ces-1*(gf) mutation is suppressed by *ced-9*(lf) mutations (Metzstein and Horvitz, 1999), consistent with *ces-1* acting upstream of *ced-9*.

Thellmann and colleagues propose that a cell's life-or-death decision is determined by a delicate balance between activators and repressors of *egl-1* expression. The results described above indicate that *ces-1* may function as a repressor of *egl-1* expression in the NSM sister cells; the authors propose that the bHLH-encoding

genes *hlh-2* and *hlh-3* may function as the activators. They show that *hlh-2* and *hlh-3* are partially required for the death of the NSM sister cells. Additionally, HLH-2 and HLH-3 are capable of binding a region of the *egl-1* promoter that is required for its expression (Thellmann et al., 2003), consistent with roles as activators of *egl-1* transcription. Interestingly, the presumed *ces-1* and *hlh-2/hlh-3* DNA binding sites overlap within the *egl-1* promoter. It is intriguing to speculate that *ces-1* and *hlh-2/hlh-3* compete for sites on the *egl-1* promoter, and that the fates of the NSM sister cells are dependent on the results of this competition. This model remains oversimplified, however. *hlh-2*(lf) and *hlh-3*(lf) mutations only partially block death of the NSM sister cells, suggesting that other genes might act redundantly with *hlh-2* and *hlh-3* to promote *egl-1* expression. Additionally, *hlh-2* is expressed in many cells in the worm (Thellmann et al., 2003), indicating that other players must confine the death-promoting activity of this gene to cells fated to die. Moreover, despite the fact that *ces-1* is broadly expressed (http://www.wormbase.org), *ces-1*(lf) mutations do not promote inappropriate cell death (Ellis and Horvitz, 1991), suggesting that other mechanisms must keep *egl-1* expression in check in cells destined for survival. Alternatively, levels of *egl-1* expression in *ces-1*(lf) animals may not be sufficient to promote the death of these cells.

Hox genes can control cell death by direct regulation of *egl-1* expression

Hox genes are required for patterning an organism along its anterior-posterior body axis, and, as such, are critical determinants of cell fate, including cell death. *C. elegans* has six characterized Hox genes, and, indeed, several of these genes are required for the appropriate specification of programmed cell death. Loss-of-function mutations in *lin-39*, the *C. elegans* homolog of *sex combs reduced*, result in the inappropriate cell death of six neurons in the midregion of the ventral nerve cord (Clark et al., 1993). Mutations in the *C. elegans antennapedia* homolog, *mab-5*, result in the inappropriate

survival of P(11/12).aaap, two cells of the posterior ventral nerve cord (Kenyon, 1986). It remains unclear whether these genes directly regulate cell death, or whether their effects on cell death are the indirect consequence of major changes in cell fate. Recent studies have demonstrated that *mab-5* may act in a complex with the homeodomain-containing protein *ceh-20* to promote P11.aaap cell death by directly activating *egl-1* transcription. Both *mab-5* and *ceh-20* are required for death of the P11.aaap cell, and for expression of an *egl-1* transcriptional reporter (Liu et al., 2006). In an approach similar to that taken by Thellmann and colleagues (2003), the authors identified a site within the *egl-1* promoter that is required for both P11.aaap cell death as well as expression of an *egl-1* transcriptional reporter, and they subsequently demonstrated that a CEH-20/MAB-5 complex is able to bind this site *in vitro* (Liu et al., 2006). Therefore, they propose that cell death in P11.aaap is specified by *mab-5*/*ceh-20*-mediated upregulation of *egl-1*.

However, several observations suggest that, while necessary for the death of P11.aaap, the upregulation of *egl-1* transcription by *mab-5* and *ceh-20* may not act as the trigger for the cell's demise. Both *mab-5* and *ceh-20* are expressed in many cells in the worm, suggesting that additional mechanisms must regulate their activity in a cell-specific manner. Moreover, while required for death of the P(11/12).aaap cells, *mab-5* expression is not sufficient for death in the lineal homologs of these cells (Salser et al., 1993). It is possible that the co-factors required for *mab-5* and *ceh-20*–induced *egl-1* expression are not expressed in other cells. Alternatively, upregulation of *egl-1* expression may not be sufficient to promote ectopic cell death in these cells.

eor-1 and *eor-2* are required for the male-specific death of the HSNs

A screen for suppressors of the ectopic cell death observed in *egl-1*(gf) mutant animals should isolate genes involved in both the execution and specification of cell death. Loss-of-function mutations in *egl-1* were isolated in this way. In addition to blocking

the death of the HSNs, *egl-1* (lf) mutations also blocked all somatic cell death (Conradt and Horvitz, 1998), thereby establishing *egl-1* as a general activator of programmed cell death. An *egl-1* (gf) suppressor screen performed by Hoeppner and colleagues isolated mutations in two more genes, *eor-1* and *eor-2*. In addition to blocking ectopic HSN cell death in *egl-1* (gf) hermaphrodites, mutations in *eor-1* and *eor-2* also blocked the death of these cells in otherwise wild-type males. Unlike the *egl-1* (lf) mutation, mutations in *eor-1* and *eor-2* do not affect programmed cell death in the pharynx, germline, or any of the other cells examined, and are therefore proposed to act in a cell-specific manner. A loss-of-function mutation in *ced-9* blocks the cell death defects observed in *eor-1* and *eor-2* mutant animals, indicating that *eor-1* and *eor-2* act upstream or in parallel to *ced-9*. The authors propose that *eor-1* and *eor-2* may normally promote the death of the male HSNs by supporting *egl-1* activity in these cells; in *egl-1* (gf) hermaphrodites, mutations in *eor-1* and *eor-2* may block HSN cell death by antagonizing ectopic *egl-1* activity.

However, how these genes interact with the cell death execution machinery, and whether they exert a direct effect on *egl-1* activity, remains unknown. *eor-1* encodes a zinc finger-containing protein with similarity to the PLZF oncogene; *eor-2* encodes a protein without any known homologues. Mutations in *eor-1* and *eor-2* result in pleiotropic defects; in addition to blocking HSN death, the mutations can also result in lethality, defects in migration of the CAN neurons, defects in male tail morphology and an inability to take up the lipophilic dye, diO (Hoeppner et al., 2004). Thus, in addition to their role in promoting HSN cell death, these two genes are likely also involved in many other neuronal cell fate decisions. These genes positively regulate expression of Ras- and Wnt-responsive genes (Howard and Sundaram, 2002; Rocheleau et al., 2002); *eor-1* and *eor-2* may act through these pathways to promote the death of the male HSNs.

Transcriptional regulation of irradiation-induced germline cell death

In addition to its prevalence in the embryo, programmed cell death is also a common cell fate in the *C. elegans* germline. Roughly half of all germ cells die via programmed cell death (Gumienny et al., 1999); animals subjected to DNA damage-inducing agents exhibit even more extensive germline cell death (Gartner et al., 2000). Unlike somatic cells, germ cells in *C. elegans* do not die in a lineage-dependent manner. As a result, work in this system has not proven useful for identifying cell-specific regulators of cell death. However, many of the players required for somatic cell death play a role in both physiological as well as DNA damage-induced germ cell death (Gumienny et al., 1999; Gartner et al., 2000), and efforts to better understand germline cell death have yielded insight into some of the mechanisms regulating the core cell death machinery. As in somatic cells, careful regulation of *egl-1* expression is critical for maintaining the balance between life and death in the *C. elegans* germline. Though not required for physiological germ cell death (Gumienny et al., 1999), *egl-1* is critical for the germ cell death that occurs in response to various DNA damaging agents (Gartner et al., 2000). In fact, levels of *egl-1* transcript in the germline, as assessed by quantitative RT-PCR, increase dramatically when animals are subjected to X-ray irradiation (Hofmann et al., 2002; Schumacher et al., 2005). This temporal correlation suggests that onset of *egl-1* expression may play a critical role in initiating germline cell death. Transcriptional upregulation of *egl-1* is dependent upon both *hus-1*, a conserved checkpoint gene required for DNA damage-induced cell cycle arrest and cell death (Hofmann et al., 2002), as well as *cep-1* (Hofmann et al., 2002; Schumacher et al., 2005), the *C. elegans* homolog of the p53 tumor suppressor (Derry et al., 2001; Schumacher et al., 2001). *ced-13*, the only other characterized BH3-only gene in *C. elegans*, is also required for DNA damage-induced germ cell death, and its expression is similarly upregulated in a *cep-1*-dependent manner upon exposure to X-ray irradiation (Schumacher et al., 2005). Intriguingly, the *ced-13* promoter

contains several *cep-1*/p53 consensus DNA binding sites (Schumacher et al., 2005). It is proposed that *cep-1* may promote germline cell death at least in part by directly activating expression of BH3-only genes.

egl-1-independent specification of cell death

The work described above demonstrates that regulation of *egl-1* expression is critical for confining activation of the cell death machinery to a specific subset of cells. However, several pieces of evidence suggest that other mechanisms of cell death specification must exist. Notably, physiological cell death in the *C. elegans* germline occurs independently of both *egl-1* (Gumienny et al., 1999) as well as the related BH3-only gene *ced-13* (Schumacher et al., 2005), suggesting that cell death specification must be regulated by other factors in this tissue. In fact, recent work by Park and colleagues (2006) suggests that the Pax transcription factors *egl-38* and *pax-2* may influence the cell death decision in both germline and somatic cells by directly regulating transcription of the *ced-9* gene. Although *egl-1* expression is necessary for cell death to occur in these cells, previous studies do not adequately address whether this expression actually triggers cell death onset. Transcription of the *egl-1* gene has not been examined at high temporal resolution, leaving open the possibility that *egl-1* expression may not provide the temporal cue regulating the initiation of cell death. Furthermore, in double mutant animals containing a strong loss-of-function mutation in *ced-9* and a very weak loss of function mutation in *ced-3*, cell death still occurs appropriately in cells destined to die and does not occur in cells destined to live (Hengartner and Horvitz, 1994). Given that *egl-1* requires *ced-9* to promote cell death, *egl-1* cannot be the determinant of cell death onset in these animals, which lack *ced-9* activity. Thus, other mechanisms controlling the timing of cell death onset must exist. The work described in this thesis demonstrates that induction of *ced-3* caspase transcription may play a critical role in controlling the onset of programmed

cell death.

Controlling the expression of cell death regulators in *Drosophila melanogaster*

Unlike death in *C. elegans* somatic cells, developmental cell death in *Drosophila melanogaster* is not predetermined by lineage, but is specified by a variety of cell-intrinsic and cell-extrinsic cues, including steroid hormone signaling, access to limiting growth factor and interaction/competition with neighboring cells. *ced-3* and *ced-4* homologs been identified in *Drosophila* (reviewed in Kornbluth and White, 2005), and studies in the fly have been critical for identifying some of the upstream signaling factors impinging upon the core cell death execution machinery. Cell death in the developing *Drosophila* embryo is activated by three genes: *reaper* (*rpr*) (White et al., 1994), *head involution defective* (*hid*) (Grether et al., 1995) and *grim* (Chen et al., 1996), which contain a shared terminal peptide RHG motif. These cell death regulators integrate various cell-death inducing stimuli, and exert their pro-apoptotic activity at least in part by promoting the auto-ubiquitination and degradation of the caspase inhibitor DIAP1 (*Drosophila* Inhibitor of Apoptosis) (Holley et al., 2002; Ryoo et al., 2002; Yoo et al., 2002; Wilson et al., 2002).

Like *egl-1*, *rpr*, *hid*, and *grim* can be regulated transcriptionally. In fact, *rpr* and *grim* are expressed uniquely in dying cells, and the onset of their expression presages cellular demise (reviewed in Tittel and Steller, 2000). *in situ* hybridization in whole mount embryos revealed that *rpr* expression precedes the onset of cell death by 1-2 hours (White et al., 1994); similar findings were observed with *grim* (Chen et al., 1996). Upregulation of *rpr* and *grim* transcript also occurs in the adult fly. Several hours post-eclosion, type II neurons, believed to play a role in wing-expansion behavior and eclosion, die via programmed cell death; *rpr* and *grim* transcript begin to be detected in these cells several hours post-eclosion, anticipating overt signs of cell death by 2-3 hours (Robinow et al., 1997). *hid* expression, on the other hand,

is not limited to cells destined to die (Grether et al., 1995), and post-translational modification must therefore be critical for regulating its death-promoting activity. Indeed, the Ras/MAPK survival pathway has been proposed to block cell death via transcriptional as well as post-translational inhibition of *hid* activity (Kurada and White, 1998; Bergmann et al., 1998). MAPK-mediated *hid* phosphorylation is critical for survival of Drosophila midline glia (Bergmann et al., 2002), where *hid* is highly expressed (Kurada and White, 1998).

Diverse cellular cues regulate the expression of *rpr*, *hid* and *grim*, thereby exerting their influence upon the cell death decision process. *rpr* expression can be induced upon exposure to various injurious insults, including X-ray irradiation (White et al., 1994; Nordstrom et al., 1996) and aberrant development (Nordstrom et al., 1996). Upregulation of *rpr* expression is also required for formation of segmental boundaries during early Drosophila development. The Hox gene *Dfd* activates cell death in some cells by directly upregulating *rpr* expression, thus ensuring formation of a proper segmental boundary between the maxillary and mandibular lobes (Lohmann et al., 2002). During *Drosophila* metamorphosis, the specific destruction of the larval midgut and salivary glands is dependent on programmed cell death mediated by the steroid hormone ecdysone (reviewed in Truman et al., 1992). Temporal control of cell death is achieved by ecdysone-induced transcriptional upregulation of *rpr* and *hid* shortly before salivary gland and midgut cell death; levels of *diap1* expression are also downregulated at this time (Jiang et al., 1997).

Conservation between worm and man

With a cell count many orders of magnitude higher than that of the worm, deciphering the cell-specific regulation of programmed cell death in humans is a daunting task. In fact, cell death in mammals is thought to be at least in part a stochastic process, with the cell death decision being made based on cell-extrinsic factors, such as access

to limiting amounts of growth factor. However, as in *C. elegans* and *Drosophila*, cell-intrinsic factors also play an important role in cell death specification. In fact, many of the mechanisms described above have parallels in mammalian development. Specifically, transcriptional upregulation of vertebrate BH3-only genes is critical for promoting cell death in some cells. In mammals, there are at least ten BH3-only genes, each of which regulates cell death in different cells and in response to different triggers. At least three of these BH3-only genes, notably Noxa, Puma and Bim, are regulated transcriptionally (reviewed in Puthalakath and Strasser, 2002). Like *egl-1* and *ced-13*, Noxa and Puma are both targets of p53, and are transcriptionally upregulated in response to genotoxic stress (Oda et al., 2000; Nakano and Vousden, 2001). Bim is expressed in response to growth factor withdrawal in both neurons (Putcha et al., 2001) and leukocytes (Dijkers et al., 2000), and subsequently promotes the death of these "starving" cells. Some of the mammalian homologs of other *egl-1* regulators are also implicated in the control of programmed cell death. The mammalian *ces-1* homolog, SLUG, is expressed in hematopoietic progenitor cells, where it acts upstream of the PUMA to block death in cells exposed to radiation (Wu et al., 2005), therefore acting in a pathway identical to that described in worms. Pbx1, the human homolog of *ceh-20*, acts as an oncogene, and is commonly mutated in patients with acute lymphoblastic anemia (Kamps et al., 1991), the most common form of pediatric cancer. It is possible that Pbx1 acts in a similar manner as *ceh-20*, affecting cell death via upstream regulation of BH3-only genes.

Induction of *ced-3* transcription may regulate the timing of PCD

We have chosen to study the initiation of programmed cell death in the *C. elegans* tail-spike cell. The tail-spike cell exhibits a unique biology, and we hypothesized that it might therefore provide novel insight into the regulation of cell death onset. We report that death of this cell depends only partially on the *egl-1* gene, suggesting

that other means of exerting temporal control on the death of this cell must exist. We demonstrate that induction of *ced-3* caspase transcription, minutes before morphological features of death are evident, plays a critical role in controlling the onset of programmed cell death in the tail-spike cell. Cell-specific regulation of caspase transcription has been previously described in both vertebrate and invertebrate systems. For example, during *Drosophila* larval metamorphosis, expression of the apical caspase *Dronc* and the effector caspase *Drice* is upregulated in response to the steroid hormone ecdysone (Cakouros et al., 2002; Daish et al., 2003; Cakouros et al., 2004; Kilpatrick et al., 2005), a well known trigger of cell death. Caspase-3 is also transcriptionally upregulated in peripheral T lymphocytes and T cell hybridomas following T cell receptor signaling (Sabbagh et al., 2004; Sabbagh et al., 2005), sensitizing the cells to respond to future death-inducing insults. However, in these systems, caspase expression, while critical for the execution of cell death, occurs hours to days before cells begin to die, and cannot, therefore, be the temporal trigger for the onset of cell death. Unlike the upregulation of the *Drosophila* caspases *Dronc* and *Drice*, or the mammalian caspase-3, *ced-3* transcription is induced minutes before the tail-spike cell dies, suggesting that *ced-3* transcription may provide the temporal cue for PCD initiation.

In a genetic screen for regulators of *ced-3* expression, we identified the homeodomain-containing transcription factor *pal-1*. *pal-1* is required for both expression of *ced-3* in the tail-spike cell and tail-spike cell death, and *in vitro* studies demonstrate that PAL-1 is able to bind the *ced-3* promoter at sites that are critical for both processes, suggesting that *pal-1* directly promotes *ced-3* expression. PAL-1 protein is similar to the mammalian protein Cdx2, which promotes intestinal development, and that when mutated can result in intestinal tumors (Chawengsaksophak et al., 1997; Aoki et al., 2003; Bonhomme et al., 2003). It is possible that Cdx2 promotes vertebrate caspase expression to effect programmed cell death in the intestinal epithelium in

a manner similar to *pal-1* regulation of *ced-3* expression in *C. elegans*. Our results unveil a novel mechanism for controlling the timing of programmed cell death in *C. elegans* by transcriptional regulation of caspases. Given the conservation between *C. elegans* and mammals, we suspect that this work may shed light upon regulation of programmed cell death in mammalian systems as well.

Chapter 2

The *C. elegans* tail-spike cell as a model for studying *ced-9* and *egl-1*-independent programmed cell death

2.1 Background

Programmed cell death (PCD) can be thought of as consisting of several steps: initiation of the death program, execution of cell death, engulfment of the dying cell, and cellular degradation. While the cell death execution machinery has been well characterized in *C. elegans*, the upstream factors that activate this machinery are, for the most part, unknown. In particular, the upstream signals responsible for targeting specific cells for death, and the molecular events that determine the timing of cell death onset, remain poorly understood. Screens designed to isolate cell-specific regulators of cell death have relied upon cell-specific markers or phenotypes (Trent, 1992; Trent et al., 1983; Hoeppner et al., 2004; Liu et al., 2006). The scarcity of these markers has hampered progress, and, currently, the molecular triggers responsible for activating the cell death machinery have been characterized in only seven of the 131 somatic cells that undergo programmed cell death in *C. elegans*.

Of the 113 somatic cells in *C. elegans* that die during embryogenesis, only two persist for longer than thirty minutes before dying: the tail-spike cell (AB.p(l/r)ppppppa), and MS.pppaaa, the sister of the germline precursor cell, Z1 (Sulston and Horvitz,

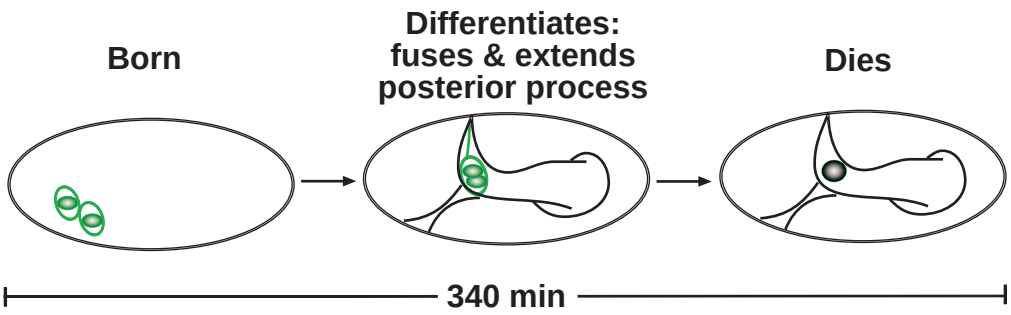

Figure 2.1: Schematic of tail-spike cell at different stages of development. Filled green ovals, tail-spike cell nuclei; gray oval, dying tail-spike cell corpse. Ball-stage embryo (left); 3-fold stage embryo (center and right).

1977; Sulston et al., 1983). To better understand how cell death is temporally regulated, we chose to investigate the mechanisms underlying the death of the tail-spike cell. Unlike most dying cells in *C. elegans*, the tail-spike cell meets its demise more than five hours after it is born (Sulston and Horvitz, 1977; Sulston et al., 1983). Whereas most dying cells in *C. elegans* are undifferentiated, the binucleate tail-spike cell exhibits extensive differentiated features before dying, including a posterior filamentous process that may function as a scaffold for modeling the *C. elegans* tail (Sulston et al., 1983) (Figure 2.1). Precise control of the timing of tail-spike cell death onset may be important for *C. elegans* tail development, and its relatively long lifespan greatly facilitates analysis of the kinetics of cell death onset. In *Drosophila* and vertebrates, many cells that die during development also exhibit obvious differentiated features, and can live long after they are born (Meier et al., 2000). Therefore, the tail-spike cell might be a useful model for understanding developmental cell death during *Drosophila* and vertebrate development. Here, we describe reagents we have developed to study tail-spike cell death, and we examine the dependence of the tail-spike cell on the previously characterized cell death machinery.

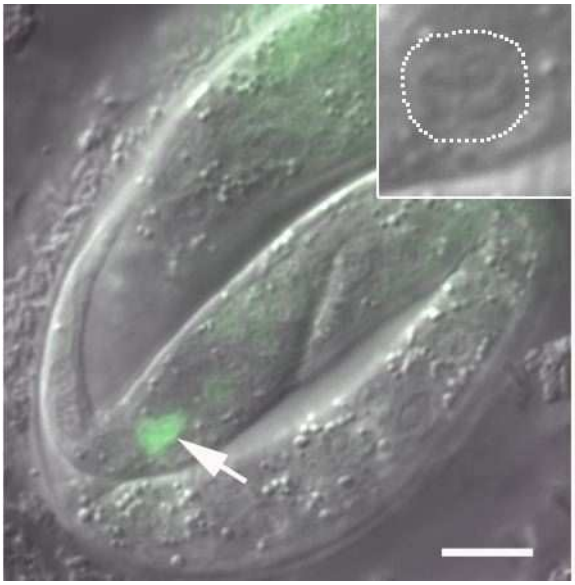

Figure 2.2: Merged Nomarski and epifluorescence image of a 3-fold stage embryo carrying a 1.5 kb *C. elegans ced-3* promoter::GFP reporter transgene (*nsEx723*). Note expression in the tail-spike cell (arrow and inset). Scale bar: $10\mu M$.

2.2 Results

Identification of a tail-spike cell reporter

Efforts to characterize the factors responsible for initiation of cell death in *C. elegans* have been hindered by the lack of cell-specific markers. The tail-spike cell provides an attractive system in part because we have developed reagents enabling us to identify the cell. We noted that a 1.5 kilobase (kb) region of the *C. elegans ced-3* promoter could promote expression of *ced-3* cDNA in the tail-spike cell (see Chapter 3). We speculated that this promoter fragment could also be used to drive reporter gene expression in the tail-spike cell, and indeed, observed that it was sufficient to drive expression of *lacZ* (Shaham, 1995; Shaham et al., 1999) or GFP (Green Fluorescent Protein) reporter transgenes in this cell (Figure 2.2). When expressed from an extrachromosomal array, however, the *C. elegans ced-3*promoter::GFP transgene occasionally blocked tail-spike cell death (Table 2.1), limiting its utility as a marker.

We reasoned that the high levels of transgene expression typical of extrachromosomal arrays might be interfering with expression from the endogenous *ced-3* promoter, thereby blocking cell death. If expressed at lower levels, the transgene might mark the tail-spike cell without affecting its death. We therefore performed low-copy integration of the *C. elegans ced-3*promoter::GFP reporter. Unfortunately, we were unable to detect GFP expression in worms from any of the ten independent transgenic lines we isolated (data not shown).

Table 2.1: *C. elegans ced-3*promoter::GFP reporter transgene blocks tail-spike cell death.

Genotype	% surviving tail-spike cells[a]	Average no. extra cells in anterior pharynx[a]
*C. elegans ced-3*promoter::GFP (*nsEx723*)	17 ± 7[b]	0.07 ± 0.26[c]
*C. elegans ced-3*promoter::GFP (*nsEx724*)[d]	0	0.14 ± 0.44
*C. briggsae ced-3*promoter::GFP (*nsIs23*)	0	0.05 ± 0.22
*C. briggsae ced-3*promoter::GFP (*nsIs25*)	0	0.5 ± 0.22

[a]For each genotype, between 20 and 30 L2 or L4 stage animals were scored for inappropriate survival of tail-spike or pharyngeal cells, respectively. [b]Standard error of the mean. [c]Standard deviation. [d]GFP expression from this transgene was very weak.

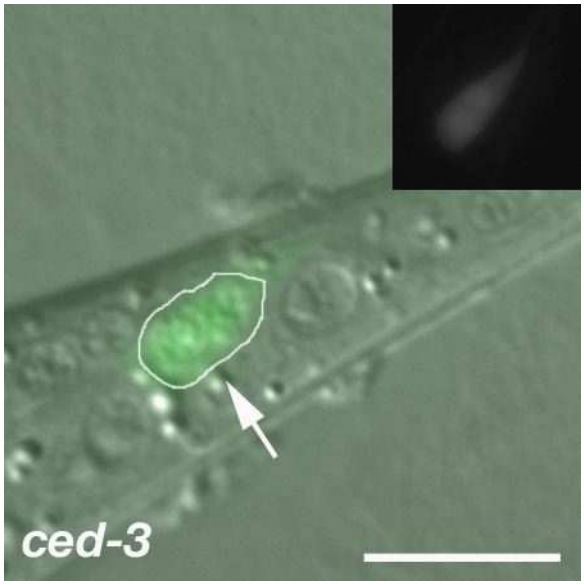

Figure 2.3: Merged Nomarski and epifluorescence image of the tail of an L2 larva carrying an integrated 0.7 kb *C. briggsae ced-3* promoter::GFP reporter construct (*nsIs25*). Note expression in the tail-spike cell (arrow; inset: GFP only). Scale bar: $10\mu M$.

As an alternate approach, we tested whether a fragment from the *ced-3* promoter of the related nematode *C. briggsae* could promote reporter expression in the tail-spike cell. Indeed, a transgene containing a 0.7 kb *C. briggsae ced-3* promoter fragment fused to the GFP-encoding gene marked the tail-spike cell (Figure 2.3), but did not promote inappropriate cell survival (Table 2.1). In animals in which tail-spike cell death was blocked, expression from both the *C. briggsae* and *C. elegans* reporters persisted in the tail-spike cell from late embryogenesis (Figure 2.2) through the larval stages (Figure 2.3) and into adulthood, allowing us to easily score larval stage animals for inappropriate tail-spike cell survival.

Table 2.2: Tail-spike cell death is dependent on *ced-3* and *ced-4*, but only partially dependent on functional *egl-1*.

Genotype[a]	% surviving tail-spike cells[b]	Average no. extra cells in anterior pharynx[b]
Wild-type	0	0.5 ± 0.2[d]
ced-3(*n717*)	100	10.6 ± 1.5
ced-4(*n1162*)	100	11.1 ± 1.1
egl-1(*n1084n3082*)	30 ± 6[c]	11.1 ± 1.5
ced-13(*tm536*)	0	0.1 ± 0.4
ced-13(*sv32*)	0	0.2 ± 0.4
egl-1(*n1084n3082*); *ced-13*(*tm536*)	41 ± 4	11.6 ± 1.4
ced-3(*n2427*)	0	2.1 ± 1.2
ced-9(*n2812*); *ced-3*(*n2427*)	0	6.5 ± 1.1
ced-3(*n2427*); *egl-1*(*n1084n3082*)	100	11.2 ± 1.8
ced-9(*n2812*); *ced-3*(*n2427*); *egl-1*(*n1084n3082*)	0	5.2 ± 1.8

[a]All strains contained either the *nsIs23* or the *nsIs25 C. briggsae ced-3* promoter::GFP integrated transgene for scoring tail-spike cell survival. *n2427* is a weak lf allele; all other alleles are nulls. [b]Between 20 and 40 L2 or L4 stage animals were scored for inappropriate survival of tail-spike or pharyngeal cells, respectively. [c]Standard error of the mean. [d]Mean ± standard deviation.

Tail-spike cell death is *ced-3* and *ced-4* dependent, but only partially dependent upon functional *egl-1*

We examined whether tail-spike cell death is dependent upon the known cell death execution machinery. Somatic cell death in *C. elegans* is governed by four genes: *egl-1*/BH3-only, *ced-9*/bcl-2, *ced-4*/Apaf-1 and *ced-3*/caspase, that function in that order in a linear pathway. Cell death is blocked by loss-of-function (lf) mutations in *ced-3*, *ced-4* and *egl-1*, and by a gain-of-function (gf) mutation in *ced-9* (Metzstein et al., 1998). As expected, we found that tail-spike cell death was completely blocked in animals homozygous for the *ced-3*(*n717*) or *ced-4*(*n1162*) null alleles (Table 2.2). Surprisingly, however, tail-spike cell death was only partially blocked in animals homozygous for the *egl-1*(*n1084n3082*) null allele, which completely blocks cell death in other somatic cells, including those of the anterior pharynx (Table 2.2). Tail-spike cell death was unaffected by null mutations in the *ced-13* gene (Table 2.2), the only other characterized *C. elegans* BH3 domain-only gene (Schumacher et al., 2005), indicating that the partial block of tail-spike cell death in *egl-1* mutants was not due to redundancy with *ced-13*.

To confirm the partial requirement for *egl-1*, we examined *egl-1*(*n1084n3082*) mutants also homozygous for the *ced-5*(*n1812*) allele. Mutations in *ced-5* block the engulfment of dying cells, resulting in persistent cell corpses (Ellis et al., 1991; Figure 2.4) which do not form if cell death is prevented (Ellis and Horvitz, 1986). We were therefore able to score tail-spike cell death without relying upon our tail-spike cell reporter. In an otherwise wild-type background, 94% of *ced-5*(*n1812*) animals exhibited a persistent tail-spike cell corpse in the first larval stage (L1). Consistent with the notion that *egl-1* only has a partial role in promoting tail-spike cell death, a majority (24 of 43, 56%) of *ced-5*(*n1812*); *egl-1*(*n1084n3082*) L1 animals still exhibited a persistent tail-spike cell corpse.

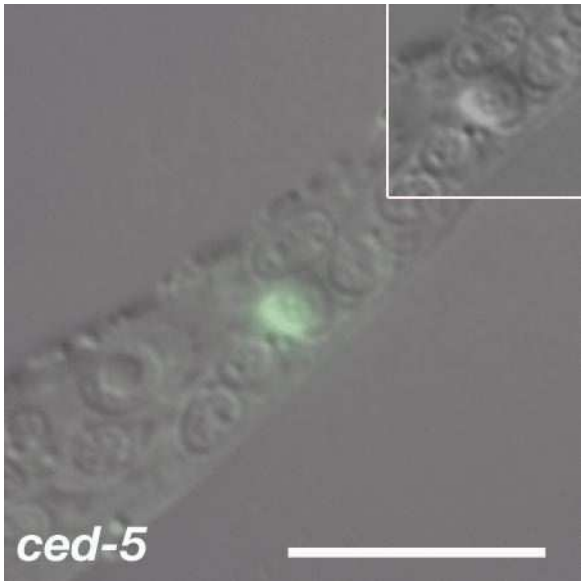

Figure 2.4: Merged GFP and Nomarski image of a distinctive tail-spike cell corpse (green) persisting in a *ced-5*(*n1812*); *nsIs25* L1 larva. Inset: tail-spike cell corpse. Scale bar: $10\mu M$.

egl-1 acts upstream of *ced-9* to promote tail-spike cell death

In most cells in *C. elegans*, *egl-1* acts upstream of *ced-9* to promote cell death (Conradt and Horvitz, 1998) by antagonizing CED-9-mediated sequestration of CED-4 (Chen et al., 2000). To determine whether, in the tail-spike cell, the pro-apoptotic activity of *egl-1* is mediated by the *ced-9* gene, we tested whether the *ced-9*(*n2812*) loss-of-function mutation could suppress the cell death defect of *egl-1*(*n1084n3082*lf) mutants. To suppress the lethality of the *ced-9*(*n2812*lf) mutation (Hengartner et al., 1992), we included the weak loss-of-function *ced-3* mutation, *n2427*, in our strains. While *ced-3*(*n2427*weak lf); *egl-1*(*n1084n3082*lf) double mutants exhibited a fully penetrant tail-spike cell death defect, *ced-9*(*n2812*lf) *ced-3*(*n2427*weak lf); *egl-1*(*n1084n3082*lf) triple mutants showed no defect in tail-spike cell death, exhibiting an identical phenotype to that observed in *ced-9*(*n2812*lf) *ced-3*(*n2427*weak lf) mutant animals (Table 2.2). Therefore, as in other cells destined to die, *egl-1* exerts its pro-apoptotic function in the tail-spike cell by inhibiting *ced-9* anti-apoptotic

functions.

Mutations in upstream regulators of *egl-1* do not affect tail-spike cell death

Several genes acting upstream of *egl-1* have been identified, among them *ces-1* and *ces-2*, which encode a Snail family member zinc finger protein (Metzstein and Horvitz, 1999; Thellmann et al., 2003) and a bZIP transcription factor (Metzstein et al., 1996), respectively. Previous studies have placed these genes in a transcriptional cascade that acts to regulate *egl-1* expression in the sister cells of the serotonergic NSM neurons of the pharynx (Ellis and Horvitz, 1991; Metzstein and Horvitz, 1999; Thellmann et al., 2003). Accordingly, *ces-2*(*n732*lf) and *ces-1*(*n703*gf) mutations block the death of these cells (Ellis and Horvitz, 1991). Neither mutation, however, affected death of the tail-spike cell (n=30).

Table 2.3: *ced-9* lacks death-promoting activity in the tail-spike cell.

Genotype[a]	% surviving tail-spike cells[b]	Average no. extra cells in anterior pharynx[b]
Wild-type	0	0.5 ± 0.2[d]
ced-3(n2427)	0	2.1 ± 1.2
ced-9(n2812); *ced-3(n2427)*	0	6.5 ± 1.1
ced-3(n2436)	59 ± 8[c]	6.3 ± 1.7
ced-9(n2812); *ced-3(n2436)*	63 ± 9	10.5 ± 1.8

[a]All strains contained either the *nsIs23* or the *nsIs25 C. briggsae ced-3* promoter::GFP integrated transgene for scoring tail-spike cell survival. *n2812* is a null allele; *n2427* and *n2436* are both weak lf alleles. [b]Between 20 and 40 L2 or L4 stage animals were scored for inappropriate survival of tail-spike or pharyngeal cells, respectively. [c]Standard error of the mean. [d]Mean ± standard deviation.

Table 2.4: *ced-9*(gf) only weakly blocks tail-spike cell death.

Genotype[a]	% surviving tail-spike cells[b]	Average no. extra cells in anterior pharynx[b]
Wild-type	0	0.5 ± 0.2[d]
ced-9(*n1950*gf)	3 ± 3[c]	10.3 ± 1.3
ced-3(*n2427*)	0	2.1 ± 1.2
ced-9(*n1950*gf); *ced-3*(*n2427*)	50 ± 9	11.5 ± 1.1
ced-3(*n2436*)	59 ± 8	6.3 ± 1.7
ced-9(*n1950*gf); *ced-3*(*n2436*)	93 ± 5	11.0 ± 1.8
ced-5(*n1812*)	3 ± 3	0.2 ± 0.5
ced-9(*n1950*gf); *ced-5*(*n1812*)	27 ± 8	10.3 ± 1.6
ced-5(*n1812*) *ced-3*(*n2427*)[e]	17 ± 7	5.1 ± 1.8
ced-9(*n1950*gf); *ced-5*(*n1812*) *ced-3*(*n2427*)[e]	73 ± 8	11.1 ± 1.5
egl-1(*n1084n3082*)	30 ± 6	11.1 ± 1.5
ced-9(*n1950*gf); *egl-1*(*n1084n3082*)	33 ± 8	10.3 ± 1.7

[a]All strains contained either the *nsIs23* or the *nsIs25 C. briggsae ced-3* promoter::GFP integrated transgene for scoring tail-spike cell survival. [b]Between 20 and 40 L2 or L4 stage animals were scored for inappropriate survival of tail-spike or pharyngeal cells, respectively. [c]Standard error of the mean. [d]Mean ± standard deviation. [e]Strain also contained the *unc-30*(*e191*) allele.

ced-9 mutations have unusual effects in the tail-spike cell

Previous studies have shown that *ced-9*(lf) mutations enhance cell survival in the pharynges of *ced-3*(weak lf) mutants (Hengartner and Horvitz, 1994), indicating that *ced-9* possesses death-promoting activity in addition to its well-characterized death-preventing activity. Surprisingly, we found that *ced-9* lacks this death-promoting activity in the tail-spike cell. Tail-spike cell death proceeded normally in *ced-9*(*n2812*lf); *ced-3*(*n2427*weak lf) double mutants (Table 2.3), and the tail-spike cell death defect in *ced-9*(*n2812*lf); *ced-3*(*n2436*weak lf) double mutants was identical to that of *ced-3*(*n2436*weak lf) single mutants (Table 2.3). We also found that, unlike other somatic cells, tail-spike cell death was only weakly affected by the *ced-9*(*n1950*) gain-of-function mutation, a glycine to glutamic acid substitution at a highly conserved *ced-9* residue (Hengartner and Horvitz, 1994). The *ced-9*(*n1950*gf) mutation alone had no effect on tail-spike cell death (Table 2.4), and it only partially enhanced the cell death defects observed in *ced-3*(weak lf) and *ced-5*(lf) backgrounds (Table 2.4). Previous studies have argued that the *ced-9*(*n1950*gf) mutation prevents cell death by disrupting a physical interaction between *ced-9* and *egl-1* (Parrish et al., 2000; Yan et al., 2004). However, such a mechanism predicts that *ced-9*(*n1950*gf) and *egl-1*(*n1084n3082*lf) mutants should exhibit identical tail-spike cell death defects. The weaker death defect observed in *ced-9*(*n1950*gf) mutant animals suggests that the *n1950*gf allele may only partially disrupt the interaction between *ced-9* and *egl-1* in the tail-spike cell, a hypothesis supported by previous biochemical studies (del Peso et al., 2000). Alternatively, the *n1950*gf allele may disrupt another aspect of *ced-9* activity.

Table 2.5: Engulfment of the tail-spike corpse is dependent on the previously characterized engulfment machinery.

Genotype[a]	% animals with persistent tail-spike corpse[b,c]	% animals with surviving tail-spike cell[c]
Wild-type	0	0
ced-1(e1755)	23 ± 8[d]	0
ced-6(n2095)[e]	17 ± 7	0
ced-7(n1892)	7 ± 4	0
ced-2(e1752)[e]	43 ± 9	3 ± 3[d]
ced-5(n1812)	93 ± 5	3 ± 3
ced-10(n1993)	27 ± 8	0
ced-12(k149)	60 ± 9	0
ced-8(n1891)	0	0

[a]All strains contained either the *nsIs23* or the *nsIs25 C. briggsae ced-3*promoter::GFP integrated transgene. [b]Tail-spike corpses were identified by position, shape, and GFP expression. [c]30 L1 stage animals per genotype were scored for defects in tail-spike cell engulfment and tail-spike cell death. [d]Standard error of the mean. [e]Strain also contained the *him-5(e1467)* allele.

Tail-spike cell engulfment is dependent upon the previously characterized engulfment machinery

In *C. elegans*, the recognition and engulfment of dying cells is dependent upon at least seven genes acting in two partially redundant signaling pathways: *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, *ced-10* and *ced-12* (Ellis et al., 1991; Hedgecock et al., 1983; Wu et al., 2001). As expected, we found that these genes are also required for engulfment of the tail-spike cell corpse (Table 2.5, Figure 2.4). Additionally, tail-spike cell death was weakly blocked by *ced-2* and *ced-5* loss-of-function mutations (Table 2.5), indicating that, as previously demonstrated (Reddien et al., 2001; Hoeppner et al., 2001), engulfment signals from neighboring cells can play a role in a cell's decision to die. Tail-spike cell death was not blocked by a loss-of-function mutation in *ced-8* (Table 2.5), which encodes a transmembrane protein involved in the kinetics of cell death (Stanfield and Horvitz, 2000). *ced-8* loss-of-function mutations result in the delayed appearance of cell corpses (Stanfield and Horvitz, 2000); however, we did not observe a persistent tail-spike corpse in *ced-8* mutant animals (Table 2.5).

2.3 Conclusions

We have established the tail-spike cell as a model for exploring the cell-specific regulation of programmed cell death. We have described the markers established to facilitate our study of tail-spike cell death, and have characterized the dependence of tail-spike cell death upon the previously characterized cell death genes. We have shown that, while tail-spike cell death is dependent upon the cell death executioner *ced-3* and its adapter gene *ced-4*, the tail-spike cell can die in an *egl-1* and *ced-9*-independent manner. Previous studies have proposed that the transcription of *egl-1* may determine the timing of cell death onset. Our results, however, suggest that other means of exerting temporal control of cell death must exist. How is cell death

initiated in the absence of *egl-1*? How is temporal control of cell death maintained in the absence of the cell death inhibitor *ced-9*? The tail-spike cell provides a tractable system to address these questions.

Chapter 3

Transcriptional induction of the *ced-3* gene plays a critical role in initiating tail-spike cell death

3.1 Background

Caspases are considered the main executioners of cell death. These proteases are synthesized as pro-proteins with weak intrinsic protease activity. Activation of their enzymatic activity is dependent upon caspase cleavage and assembly of the resulting subunits into heterodimers (reviewed in Yan and Shi, 2005). Caspase-mediated proteolytic cleavage of target substrates then triggers a cell's demise (Enari et al., 1998; Sakahira et al., 1998; Rao et al., 1996; Buendia et al., 1999). Previous studies have suggested that cell death initiation is regulated by direct or indirect control of the post-translational activation of caspases. In *C. elegans*, reports have suggested that the *ced-3* caspase is expressed in most cells, including cells fated to live (Shaham and Horvitz, 1996a). Activation of CED-3 is indirectly regulated by *egl-1* (Chen et al., 2000), consistent with the hypothesis that *egl-1* is an important trigger of cell death.

We have shown, however, that *egl-1* is only partially required for death of the tail-spike cell (see Chapter 2), suggesting that an alternate mechanism must control the initiation of programmed cell death. Such a mechanism must function upstream of or in parallel to the *ced-3* caspase, since a *ced-3*(strong lf) allele completely blocked

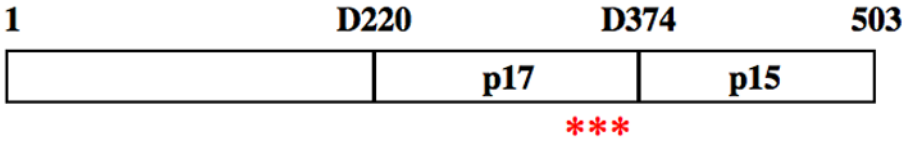

Figure 3.1: Schematic of CED-3 pro-protein. Cleavage sites are indicated (D220 and D374). Asterisks mark the region targeted by the anti-CED-3 antibody.

tail-spike cell death (see Chapter 2). Here, we provide evidence that induction of *ced-3* transcription plays a critical role in the initiation of tail-spike cell death. We show that *ced-3* transcription is induced in the tail-spike cell shortly before it dies, and we identify regulatory sites within the *ced-3* promoter that are specifically required for both *ced-3* expression in the tail-spike cell as well as tail-spike cell death.

3.2 Results

Hints that *ced-3* expression is regulated differently in the tail-spike cell

Unexpectedly, several lines of evidence implicated transcription of *ced-3* as a potential step in regulating the timing of tail-spike cell death. Initially, we noticed that *ced-3* expression was modulated differently in the tail-spike cell as compared to other dying cells. As described in Chapter 2, a 1.5 kb *C. elegans ced-3* promoter::GFP reporter occasionally blocked tail-spike cell death, possibly by titering a limiting transcription factor. This reporter had no effect on other cell deaths we scored. Furthermore, while expression from *ced-3* promoter::GFP transgenes persisted through adulthood in tail-spike cells of *ced-3* mutants (see Chapter 2), expression was not observed in other inappropriately surviving cells (data not shown).

Attempts to directly follow *ced-3* expression have been unsuccessful

To examine whether *ced-3* expression could be important in regulating the timing of tail-spike cell death onset, we sought to follow the kinetics of *ced-3* expression in the tail-spike cell in greater detail. Unfortunately, RNA *in situ* hybridization cannot be used reliably to detect *ced-3* expression (S. Shaham, unpublished). Our attempts to generate an antibody to recognize CED-3 were also unsuccessful. Specifically, we generated antibodies in rats against a 15-amino acid peptide corresponding to the C-terminal region of the CED-3 large subunit (Figure 3.1). We operated under the assumption that the N and C termini of CED-3 peptides may become exposed upon CED-3 activation and cleavage; structural studies have suggested that this is indeed the case with the CED-3 mammalian homolog, caspase-3 (Shi, 2002). A similar approach had previously been used to generate antibodies to human/mouse caspase-3 (Srinivasan et al., 1998). We tested the specificity of our antibodies on embryos carrying a transgene expressing CED-13/BH3-only under the control of a heat-shock inducible promoter. Expression from this transgene results in extensive cell death that is dependent upon *ced-3* (Schumacher et al., 2005). We failed to detect immunostaining with our anti-CED-3 antibodies in embryos expressing this transgene. Antisera generated against human/mouse caspase-3 (CM1; Srinivasan et al., 1998), *Drosophila* full-length Drice, or *Drosophila* activated Drice also failed to immunostain these embryos.

ced-3 transcription is induced shortly before tail-spike cell death onset

Given that we could not directly follow production of either *ced-3* mRNA or CED-3 protein, we decided to follow *ced-3* expression kinetics indirectly in embryos carrying either the *C. elegans* or *C. brigssae ced-3* promoter::GFP reporters (lines *nsEx723* and *nsIs25*, respectively). Intriguingly, while expression of either reporter was detected in many cells, and throughout embryogenesis (data not shown), expression in the

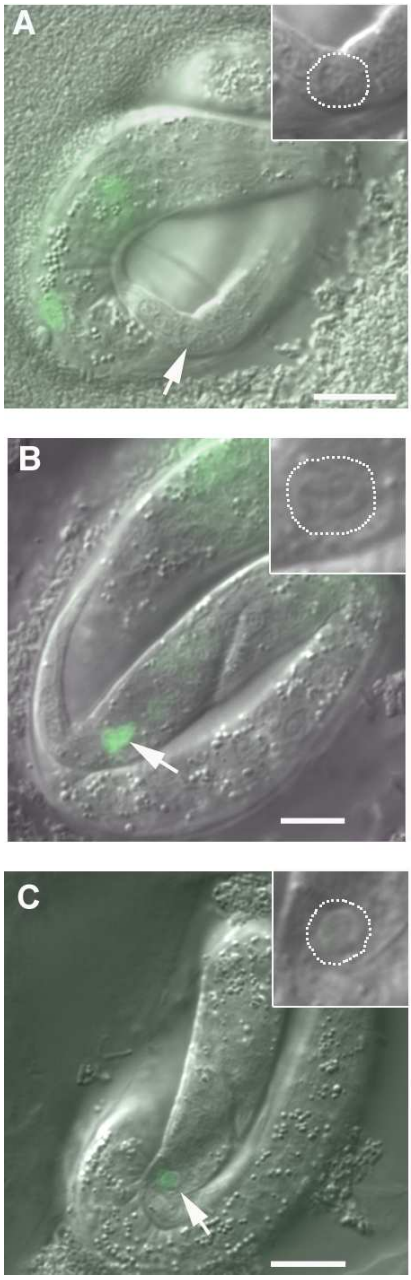

Figure 3.2: Expression of *ced-3* promoter::GFP transgenes is induced shortly before tail-spike cell death. Merged Nomarski and epifluorescence images of 3-fold stage embryos carrying a 1.5 kb *C. elegans ced-3* promoter::GFP reporter transgene (*nsEx723*); (A) embryo before onset of tail-spike cell GFP expression (arrow and inset: binucleate tail-spike cell), (B) embryo at onset of tail-spike cell GFP expression (arrow and inset: binucleate tail-spike cell), and (C) embryo approximately 31 minutes after onset of tail-spike cell GFP expression (arrow and inset: dying tail-spike cell corpse). Scale bar: $10\mu m$.

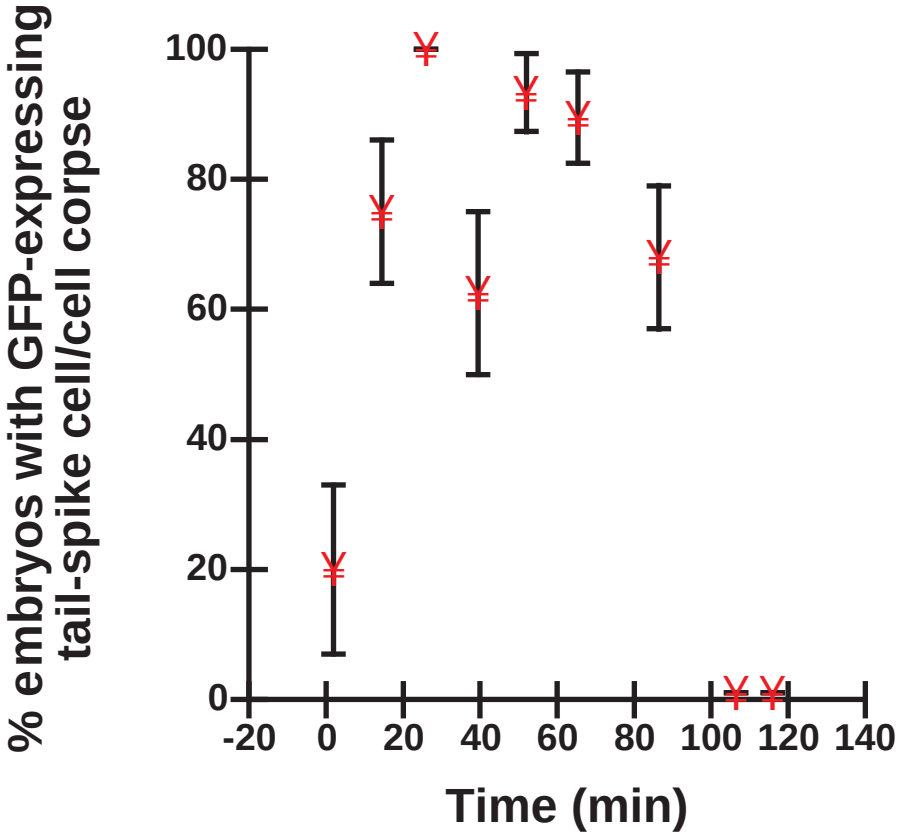

Figure 3.3: Population study quantifying duration of tail-spike cell GFP expression in animals carrying a 0.7 kb *C. briggsae* promoter::GFP reporter transgene (*nsIs25*). See Chapter 8 for experimental details. Error bars: standard error of the mean.

tail-spike cell was only observed during the three-fold stage of embryogenesis (Figure 3.2), hours after the tail-spike cell is born. We followed individual embryos through this stage, and found, surprisingly, that GFP fluorescence in the tail-spike cell first appeared 32.1 ± 4.9 minutes (mean $\pm$ SD; n=6; *C. elegans* reporter), or 24.8 ± 2.5 minutes (n=5; *C. briggsae* reporter), prior to visible signs of cell death (Figure 3.2). Similar findings were obtained by scoring *ced-3* expression at specific time points in populations of synchronized embryos (Figure 3.3). That multiple lines carrying *C. elegans* and *C. brigssae* reporter transgenes, as well as multiple derivatives of the *C. elegans* transgenes (see below), exhibited identical kinetics of *ced-3* expression in the tail-spike cell suggests that these reporters are likely to faithfully represent endogenous expression of *ced-3*.

To further support this notion, we examined whether the same *C. elegans ced-3* promoter used in our GFP reporter construct could promote tail-spike cell death in *ced-3*(*n717*) mutants when used to drive expression of a *ced-3* cDNA. Indeed, we found that this construct was sufficient to promote tail-spike cell death in six independent transgenic lines (Figure 3.4), indicating that *ced-3* expression at this late stage in the development of the tail-spike cell is sufficient to promote the cell's demise, and supporting the hypothesis that *ced-3* transcription may be the temporal trigger for cell death initiation in the tail-spike cell. Such a mechanism for cell death initiation has not been previously described. Interestingly, the expression construct did not promote cell death in cells of the anterior pharynx (Figure 3.4), suggesting that *ced-3* expression may be regulated differently in these cells.

Regulatory regions within the *ced-3* promoter

To further investigate whether *ced-3* transcription plays a role in tail-spike cell death, we examined *ced-3* promoter function in greater detail. The *C. elegans ced-3* promoter contains a 349 bp sequence that is conserved in *C. briggsae* (Figure 3.5), and

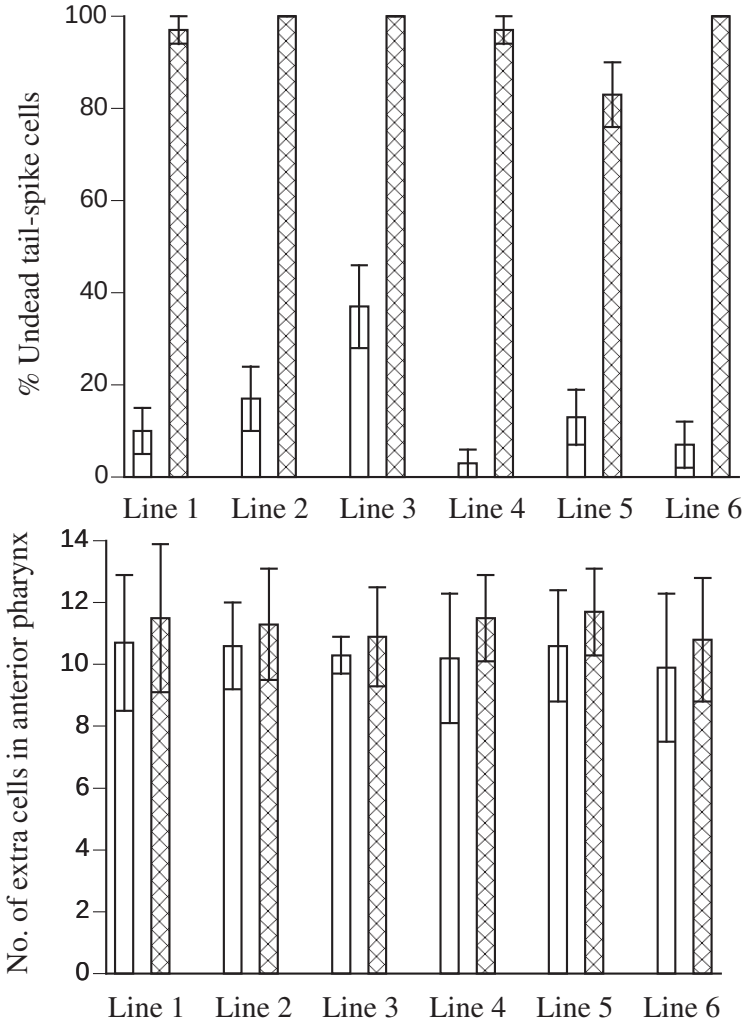

Figure 3.4: 1.5 kb *C. elegans ced-3* promoter is sufficient to promote tail-spike but not pharyngeal cell death when upstream of *ced-3* cDNA. For each transgenic line, between 20 and 30 L3 stage *nsIs25*; *ced-3*(*n717*) animals carrying a transgene containing a *ced-3*promoter::cDNA construct were scored for inappropriate survival of tail-spike (A) or pharyngeal cells (B). White columns: animals expressing transgene; hatched columns: animals not expressing transgene. Error bars: standard error of the mean (tail-spike cell), standard deviation (pharyngeal cells). Note: To rule out any effects of the rescue construct on reporter expression, ten 3-fold stage embryos from line 6 were examined using a fluorescence-equipped compound microscope. Tail-spike cell reporter expression was wild-type in animals carrying the rescue construct.

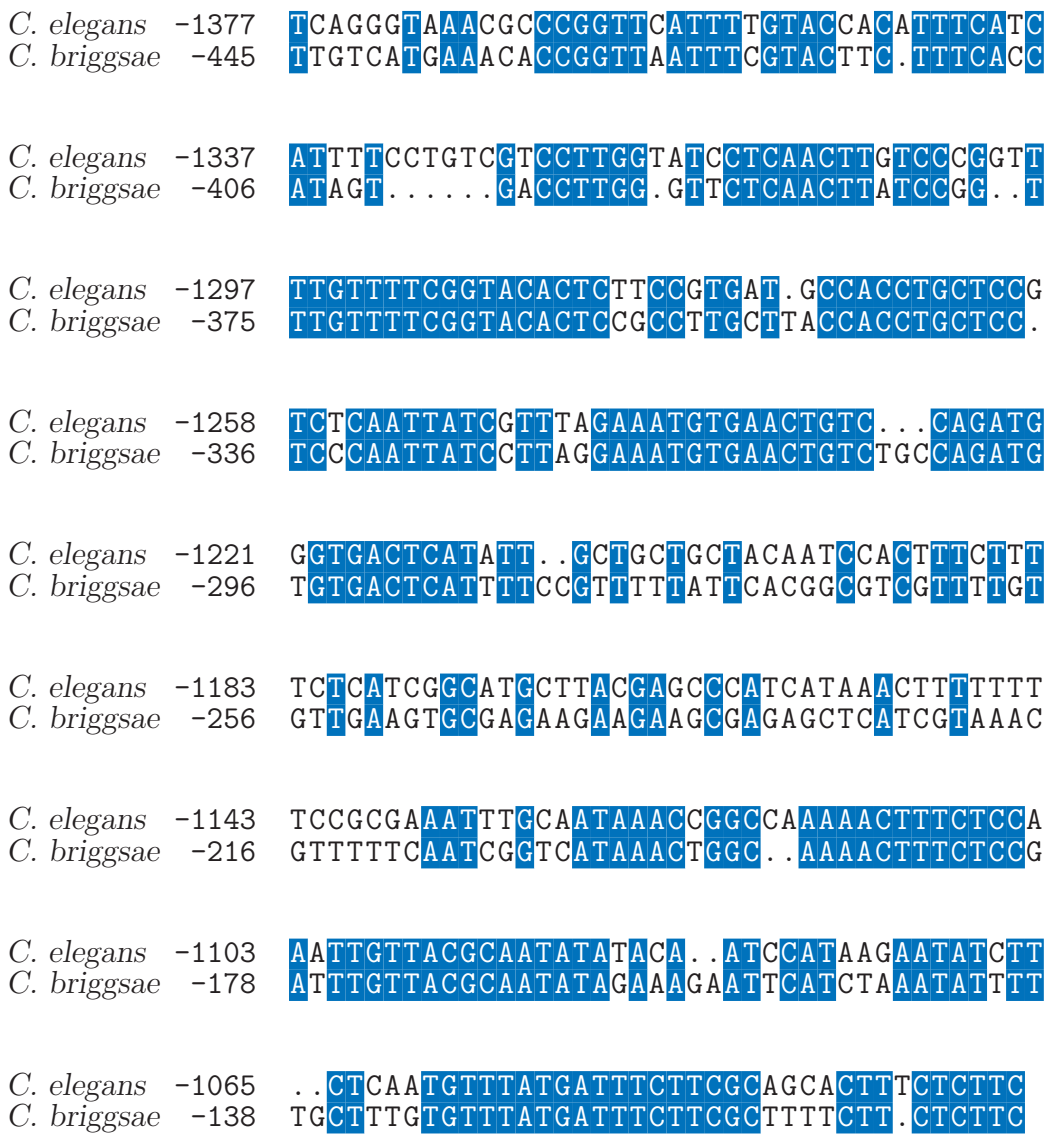

Figure 3.5: Alignment of the conserved region of the *ced-3* promoter of *C. elegans* and *C. briggsae*; identical residues are shaded. Numbering is relative to the *ced-3* start codon.

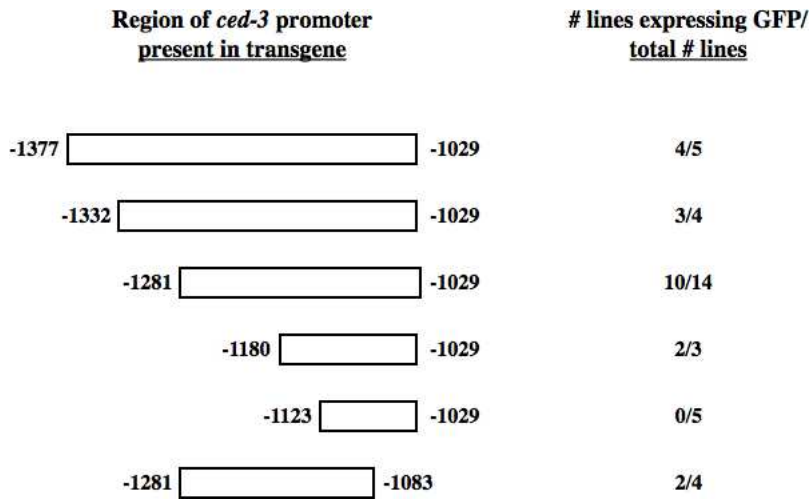

Figure 3.6: Truncated regions of the *C. elegans ced-3* promoter were tested for their ability to drive GFP reporter expression in the tail-spike cell. Numbering is relative to the *ced-3* start codon. At least 15 animals were examined per transgenic line.

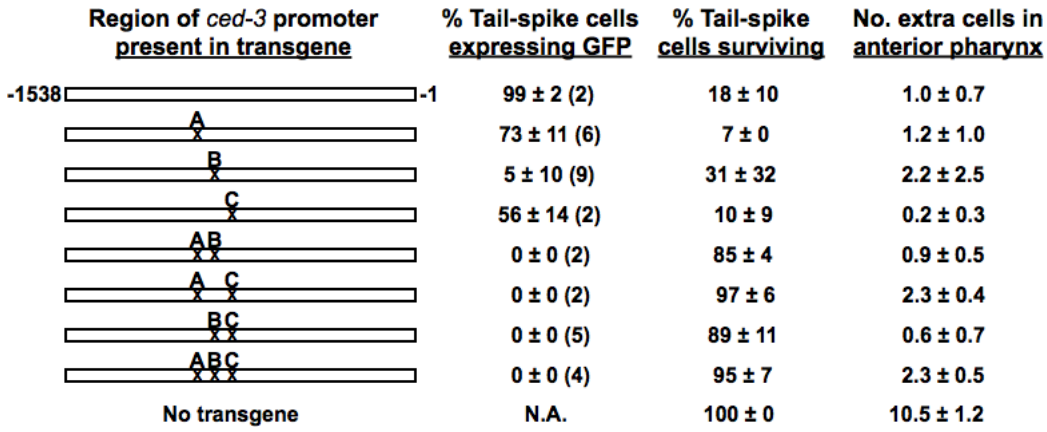

Figure 3.7: Regions A, B, and C are required for GFP reporter expression and for rescue of the *ced-3*(*n717*) cell death defect in the tail-spike cell. Regions A (-1198 to -1183), B (-1157 to -1142), or C (-1226 to -1113) were deleted in the context of the 1.5 kb *C. elegans ced-3* promoter (Column 1), or pJ40, a plasmid containing *C. elegans ced-3* genomic DNA (Columns 2 and 3). Constructs were introduced into *ced-3*(*n717*) worms, and animals were assessed for tail-spike cell reporter expression (Column 1) and tail-spike and pharyngeal cell death defects (Columns 2 and 3, respectively). x, deleted region; % Tail-spike cells expressing GFP: average ± standard error (No. transgenic lines examined); % Tail-spike cells surviving: average ± standard error (2-3 transgenic lines); No. extra cells in anterior pharynx: average ± SD (2-3 transgenic lines). N.A.: not applicable.

Deleted region of *ced-3* promoter [a]	No. transgenic lines expressing GFP / total no. lines[b]
-1332 to -1318	3/3
-1317 to -1303	2/2
-1287 to -1273	1/2
-1272 to -1258	6/6
-1257 to -1243	6/6
-1242 to -1228	2/2
-1227 to -1213	1/1
-1212 to -1199	2/3
-1198 to -1183	**0/9**
-1186 to -1171	5/5
-1170 to -1158	5/5
-1157 to -1142	**0/6**
-1151 to -1127	5/6
-1226 to -1113	**0/6**
-1117 to -1100	4/4
-1104 to -1085	2/2
-1092 to -1069	3/3
-1071 to -1054	7/7
-1053 to -1039	5/5
-1038 to -1028	2/2

Table 3.1: Three regions of the *C. elegans ced-3* promoter are required for tail-spike cell expression of a 349 bp *C. elegans ced-3*::GFP reporter. [a]Numbering is relative to the *ced-3* start codon. [b]At least 15 animals were examined per transgenic line.

that is sufficient to drive GFP reporter expression in the tail-spike cell (see Chapter 2). In fact, we found that promoter stretches containing as few as 150 bp from this well-conserved region were sufficient to promote tail-spike cell reporter expression (Figure 3.6). To identify specific regions within the conserved promoter required for *ced-3* expression in the tail-spike cell, we deleted consecutive 14-16 bp sequences within this promoter, and assessed the effects of these deletions on reporter transgene expression. As shown in Table 3.1 and Figure 3.7, three regions, A (-1199 to -1183), B (-1157 to -1142), and C (-1226 to -1113), were required for *ced-3* promoter::GFP expression in the tail-spike cell. The sequences of regions B and C are highly conserved in *C. briggsae* (Figure 3.8). Although deleting regions A, B, or C individually

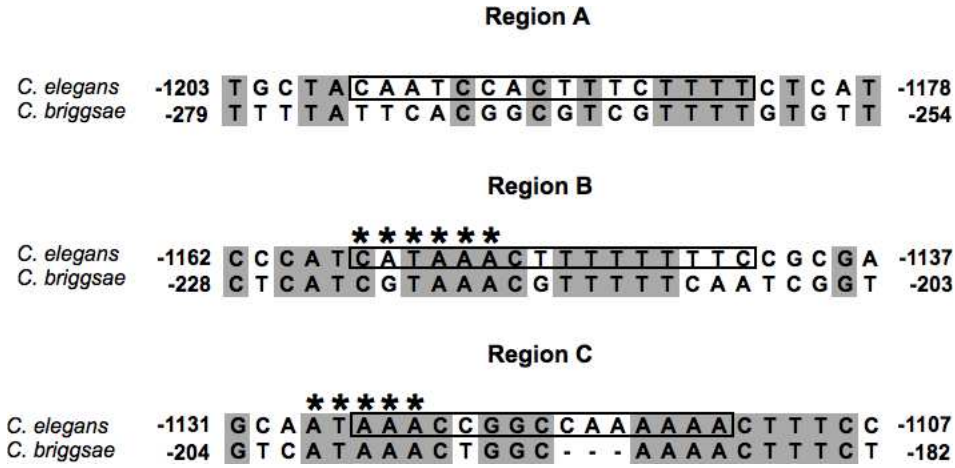

Figure 3.8: Alignment of the three *ced-3* promoter regions (A, B, and C) from the nematodes *C. elegans* and *C. briggsae*. Conserved nucleotides are shaded; boxed nucleotides are deleted in Table 3.1 and Figure 3.7; consensus nucleotides of caudal/Cdx2 binding sites (see Chapter 4) are indicated by asterisks above the relevant nucleotides; numbers indicate positions relative to the *ced-3* start codon.

in the larger 1.5 kb *ced-3* promoter::GFP transgene did not fully block GFP expression (a significant partial effect was seen upon deletion of region B), double deletions abolished GFP expression in the tail-spike cell (Figure 3.7), suggesting that these sequences function redundantly to control *ced-3* expression in this cell. On their own, tandem copies of sites A, B or C were insufficient to drive GFP reporter expression in the tail-spike cell (data not shown), suggesting that these sites, while necessary, are not sufficient to promote *ced-3* expression in the tail-spike cell.

To assess the functional relevance of these redundant promoter sequences, we deleted them singly or in combination in the context of a 7.6 kb rescuing *C. elegans ced-3* genomic clone containing the same 1.5 kb of 5' promoter sequences (Yuan et al., 1993). These clones were individually introduced into *ced-3* mutants, and their ability to rescue the cell death defect was assessed. We found that transgenes lacking two or more of these sites did not restore tail-spike cell death, but rescued inappropriate cell survival in the anterior pharynx (Figure 3.7), indicating that the sites are required specifically for tail-spike cell death. Taken together, the expression and rescue results

strongly suggest that induction of *ced-3* transcription is a key step in promoting the initiation of tail-spike cell death.

Table 3.2: In vivo RNAi directed against *ced-3* has a weak effect on tail-spike cell death, and no effect on cell death in the anterior pharynx.

Tail spike cell survival

Transgenic Line	0 hours[a]	2 hours[a]	4 hours[a]	6 hours[a]
Line 1				
+ *ced-3* IR	7 ± 5[b]	17 ± 7	13 ± 6	3 ± 3
− *ced-3* IR	5 ± 5	0	0	4 ± 4
Line 2				
+ *ced-3* IR	0	7 ± 5	0	0
− *ced-3* IR	0	0	0	0
Line 3				
+ *ced-3* IR	4 ± 4	60 ± 9	13 ± 6	0
− *ced-3* IR	0	0	0	0

Survival of cells in anterior pharynx

Transgenic Line	0 hours[a]	2 hours[a]	4 hours[a]	6 hours[a]
Line 1				
+ *ced-3* IR	0.07 ± 0.3[c]	0.2 ± 0.4	0.2 ± 0.4	0.03 ± 0.2
− *ced-3* IR	0	0.05 ± 0.2	0.04 ± 0.2	0.05 ± 0.2
Line 2				
+ *ced-3* IR	0.1 ± 0.3	0.3 ± 0.5	0.06 ± 0.3	0
− *ced-3* IR	0	0.05 ± 0.2	0.05 ± 0.2	0.1 ± 0.3
Line 3				
+ *ced-3* IR	0.1 ± 0.3	0.5 ± 0.5	0.3 ± 0.5	0.1 ± 0.3
− *ced-3* IR	0	0.3 ± 0.5	0.3 ± 0.5	0.2 ± 0.4

Inappropriate tail-spike and pharyngeal cell survival was assessed in L4 stage animals that had been subjected to heat shock at the indicated times. Animals with (+) or without (−) the *ced-3* IR gene were scored. [a]Approximate age (number of hours post-fertilization) of embryos at onset of heat shock. Age is a rough approximation, and assumes that embryos were laid 2 hours post-fertilization. [b]Percentage of progeny with inappropriately surviving tail-spike cell ± standard error of the mean. [c]Average number of extra cells in the anterior pharynx per animal ± standard deviation. Note: in the absence of heat-shock, the *ced-3* IR transgene did not block tail-spike or pharyngeal cell death (assessed in at least 15 animals per line).

Attempts to block *ced-3* expression via *in vivo* RNAi were unsuccessful

To test whether tail-spike cell death is dependent upon induction of *ced-3* transcription, we performed *in vivo* RNAi. Previous studies have demonstrated that *in vivo* expression of double stranded (ds) RNA can mediate specific, inducible gene silencing in *C. elegans*. RNA containing an inverted repeat (IR) can be processed into a hairpin structure, which may activate a cell's endogenous RNAi machinery and selectively downregulate gene expression (Tavernarakis et al., 2000). We used this approach to knockdown *ced-3* expression at specific times during embryogenesis. Specifically, we expressed an inverted repeat "gene" containing 2 inverted copies of the *ced-3* coding sequence under the control of the heat shock-inducible promoters *hsp16-2* and *hsp16-41*. Expression of the *ced-3* IR was induced in embryos ranging in age from zero to six hours post-fertilization. (For reference, the tail-spike cell dies approximately ten hours post-fertilization, and cells of the pharynx die between six to seven hours post-fertilization.) For the most part, expression of the *ced-3* IR had little or no effect on tail-spike or pharyngeal cell death (Table 3.2). Intriguingly, however, the *ced-3* IR blocked tail-spike cell death most effectively when expressed several hours before the tail-spike cell dies (Table 3.2). This may suggest that tail-spike cell death is dependent on *ced-3* expression during early embryogenesis. However, the results from this experiment are difficult to interpret for several reasons. First, we do not know the interval between induction of the *ced-3* IR and effective knockdown of gene expression. Expression of *ced-3* IR did not block pharyngeal cell death (Table 3.2), suggesting that, after its expression, several hours may be required to properly fold the *ced-3* IR into a *ced-3* hairpin structure. Additionally, induction of the *ced-3* IR in L4 stage animals had no effect on tail-spike or pharyngeal cell death of their progeny in any of three independent transgenic lines examined, the opposite result from that described by Tavernarakis et al. (2000). This discrepancy suggests that the *ced-3* hairpin structure may be unstable, and also demands that our results and

experimental protocol be examined with greater scrutiny.

Sequencing previously isolated alleles of *ced-3*

The similarity between the *C. elegans* and *C. briggsae* promoters (Figure 3.5) is highly suggestive of functional relevance. Indeed, we have demonstrated that three short stretches of this promoter are required for *ced-3* expression in the tail-spike cell. We took an indirect approach to test whether sites within this promoter also play a role in other cell deaths in *C. elegans*. In particular, we asked whether previously isolated *ced-3* alleles contain mutations within this conserved region of the *ced-3* promoter. Alleles *n2428*, *n2448*, *n2875*, *n2869*, *n2920*, *n2855*, and *n2455* have been previously classified as *ced-3* alleles by their failure to complement *ced-3*(*n717*) (S. Shaham, unpublished results); however, their mutations have not yet been identified. We reasoned that these mutations might reside within regulatory regions of the *ced-3* promoter; in fact, *n2869* mutants, which possess a significant cell death defect (8.8 ± 1.4 extra cells in the pharynx, S. Shaham, unpublished results), do not contain mutations within the *ced-3* open reading frame (S. Shaham, unpublished results). We did not, however, detect mutations within the conserved region of the *ced-3* promoter (-1356 to -1029) in any of the alleles. Other approaches will need to be taken to further assess the role of the conserved *ced-3* promoter in regulating cell death outside the tail-spike cell. For example, some of the same approaches aimed at identifying regulatory sites for the tail-spike cell may be useful for identifying sites required for *ced-3* expression elsewhere in the worm.

3.3 Conclusions

We have previously shown that the *egl-1* and *ced-9* genes, which are required for the majority of somatic cell deaths in *C. elegans*, only play a minor role in the death of the tail-spike cell (see Chapter 2), suggesting that another pathway must exist that

specifically regulates the death of this cell. Here, we have provided evidence suggesting that regulation of tail-spike cell death may be achieved through transcriptional control of the *ced-3* gene. We have demonstrated that, in the tail-spike cell, *ced-3* caspase is expressed minutes before the cell displays obvious signs of death, and that this induction is sufficient for the cell's demise. We have also identified specific sites within the *ced-3* promoter that are required both for *ced-3* expression in the tail-spike cell and for tail-spike cell death. These findings imply that induction of *ced-3* transcription is required for initiation of tail-spike cell death.

Our observations thus far suggest the following model: in the tail-spike cell, *egl-1* and *ced-9* may have attenuated function, thus allowing CED-4 protein to remain unchecked. In the absence of CED-3 caspase, CED-4 is unable to promote cell death, thus allowing the tail-spike cell to live. Upon transcription of *ced-3*, accumulating CED-3 protein may become immediately processed through interactions with CED-4, leading to rapid killing of the cell. The subsequent chapter will address how the transcription of *ced-3* is regulated.

Chapter 4

The homeodomain-containing transcription factor *pal-1* promotes tail-spike cell death by upregulating *ced-3* transcription.

4.1 Background

Previous studies, both in *C. elegans* as well as in vertebrates, have suggested that the components of the cell death execution machinery, including caspases, are constitutively expressed in most, if not all, cells, and are present at levels that are sufficient to execute a cell's death program (Shaham and Horvitz, 1996a; Weil et al., 1996). We have demonstrated, however, that *ced-3* transcription is induced very late in the life of the *C. elegans* tail-spike cell; expression is observed only minutes before morphological features of death are evident in this cell (Chapter 3). The tight temporal correlation between *ced-3* expression and cell death in the tail-spike cell suggests that *ced-3* transcription may provide the temporal cue for initiating the cell's death program. To further explore this model, we performed a forward genetic screen designed to identify regulators of *ced-3* expression in the tail-spike cell. The results of this screen are described below. Most notably, we isolated two mutations in the homeodomain-containing transcription factor *pal-1*. We describe the characterization of these mutants, and report the results of experiments establishing *pal-1* as a direct regulator of *ced-3* expression.

4.2 Results

Isolating mutants defective in *ced-3* transcription

To identify regulators of *ced-3* expression in the tail-spike cell, F2 progeny of mutagenized *ced-3*(*n717*) carrying the *C. briggsae ced-3*promoter::GFP reporter (*nsIs25*) were screened for loss of tail-spike cell GFP expression. From 64,000 haploid genomes examined, we recovered three independent mutants (*ns90*, *ns114*, and *ns115*) defective in tail-spike cell GFP reporter expression. All three mutants exhibited a wild-type reporter expression pattern in other cells examined (data not shown). Both *ns114* and *ns115* mutant animals possessed partially penetrant defects in reporter expression. *ns114* and *ns115* heterozygous mutant animals exhibited wild-type tail-spike cell GFP expression (*ns114*: 47/47; *ns115*: 25/25), indicating that these mutations act recessively to block reporter expression in the tail-spike cell. Characterization of these mutants is described below. The *ns90* mutation resulted in a complete block of *ced-3*promoter::GFP expression in the tail-spike cell (39/39 L2 stage animals). *ns90* heterozygous mutant animals were also defective in tail-spike cell reporter expression (24/25, 96%). The *ns90* mutation could therefore be acting semi-dominantly, or a maternal contribution of the gene product mutated by *ns90* could be required. 42 of 62 (68%) of the progeny of *ns90* heterozygotes lacked reporter expression in the tail-spike cell, indicating that *ns90* may be acting semi-dominantly to block *C. briggsae ced-3*promoter::GFP expression. We mapped *ns90* to a region of chromosome II between cosmids T10D4 (-8.3441) and C50E10 (8.2062) (data not shown). Cloning of the gene mutated in *ns90* mutant animals, and more extensive characterization of the *ns90* mutant phenotype, remain to be performed.

To introduce mutations, we used the mutagen ethyl methanesulfonate (EMS). In *C. elegans*, mutagenesis with EMS at a concentration of 50 mM typically generates a loss of function mutation affecting the average sized gene at a rate of 1×10^{-4}

to 5×10^{-4} per mutagenized gamete. To ensure saturation, we performed three rounds of screening, examining a total of 32,000 F2s, or 64,000 haploid genomes. As described below, we isolated two independent mutations in the same gene, suggesting that the screen was performed to saturation. However, it is possible that some of the genes required for tail-spike cell *ced-3* expression are also required for general development; mutations in such genes might result in embryonic lethality, sterility, or larval arrest, and would therefore not be isolated in a non-clonal screen. Of the 621 candidate mutants we isolated, only 130 (21%) generated progeny, suggesting that some of the factors governing *ced-3* expression may also be required for organismal survival. Alternatively, it is also possible that the high lethality we observed was non-specific. In fact, a comparable fraction of mutagenized animals with wild-type reporter expression (24/30) failed to generate progeny.

Mutations reducing *ced-3* transcription also block cell death

ns114; *ced-3*(*n717*) animals exhibited weak (42%, n=88, 3 lines scored) or absent (58%) *C. elegans ced-3*promoter::GFP expression in the tail-spike cell. Similar results were obtained using the *C. briggsae* reporter transgene *nsIs25* (Figure 4.1). We hypothesized that *ns114* might also block tail-spike cell death. Two observations suggested that this was indeed the case. First, in an otherwise wild-type genetic background, 16 of 75 (21%) *ns114*; *nsIs25* animals had an inappropriately surviving tail-spike cell, as scored by weak GFP expression in this cell. By contrast, cell death in the anterior pharynx was unaffected (0.06 ± 0.08 extra cells in the anterior pharynx, average $\pm$ standard deviation, $n = 29$). Identifying inappropriately surviving tail-spike cells by reporter expression alone underestimates the cell death defect in *ns114* mutant animals, since about 50% of tail-spike cells do not express GFP at all. As an alternate approach, we assayed the *ns114* cell death defect in a *ced-5* background. Whereas 94% of *ced-5*(*n1812*) animals exhibited a persistent tail-spike cell corpse

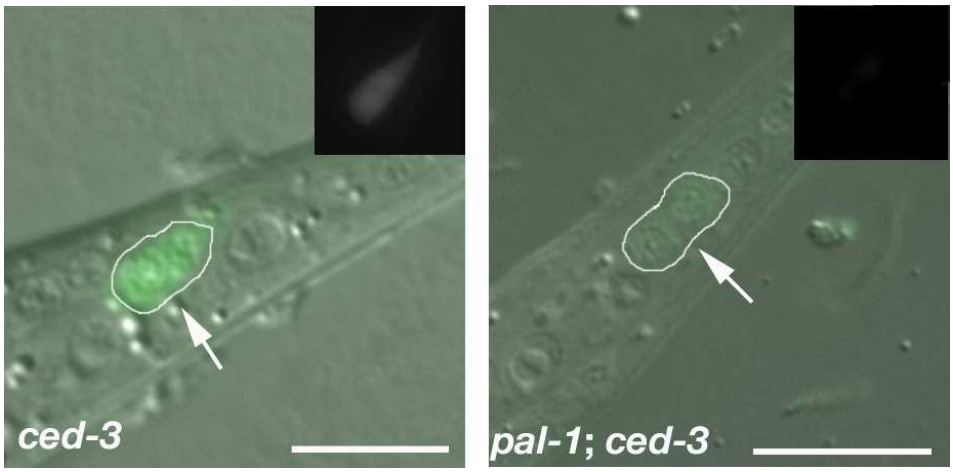

Figure 4.1: The *ns114* mutation blocks *ced-3* promoter::GFP reporter expression. Merged Nomarski and epifluorescence images of the tails of *ced-3*(*n717*) and *pal-1*(*ns114*); *ced-3*(*n717*) L2 larvae (left and right, respectively) carrying a 0.7 kb *C. briggsae ced-3*promoter::GFP reporter transgene (*nsIs25*). Images were shot using the same exposure time; note that reporter expression is significantly diminished in the tail-spike cell (arrow and inset) of the *pal-1*(*ns114*); *ced-3*(*n717*) animal as compared to that of the *ced-3*(*n717*) animal. Scale bar: 10μm.

(see Chapter 2), a persistent cell corpse was only seen in 20 of 46 (43%) *ns114*; *ced-5*(*n1812*) animals (Figure 4.2), further indicating that the *ns114* mutation partially blocked tail-spike cell death. Interestingly, the tail-spike cell death defect was greatly enhanced in *ns114*; *egl-1*(*n1084n3082*) double mutants compared to either single mutant alone (Figure 4.2), suggesting that *ns114* may affect a gene acting in parallel to *egl-1* to promote tail-spike cell death. Consistent with this result, 10/32 (31%) *ns114*; *ced-9*(*n2812*lf); *ced-3*(*n2427*) animals had inappropriately surviving tail-spike cells, as scored in cells weakly expressing GFP. Tail-spike cell death proceeds normally in *ced-9*(*n2812*lf); *ced-3*(*n2427*) animals (n=20), indicating that *ns114* may affect a gene that functions in parallel to *ced-9*.

In addition to defects in *ced-3* expression and tail-spike cell death, 27 of 30 (85%)

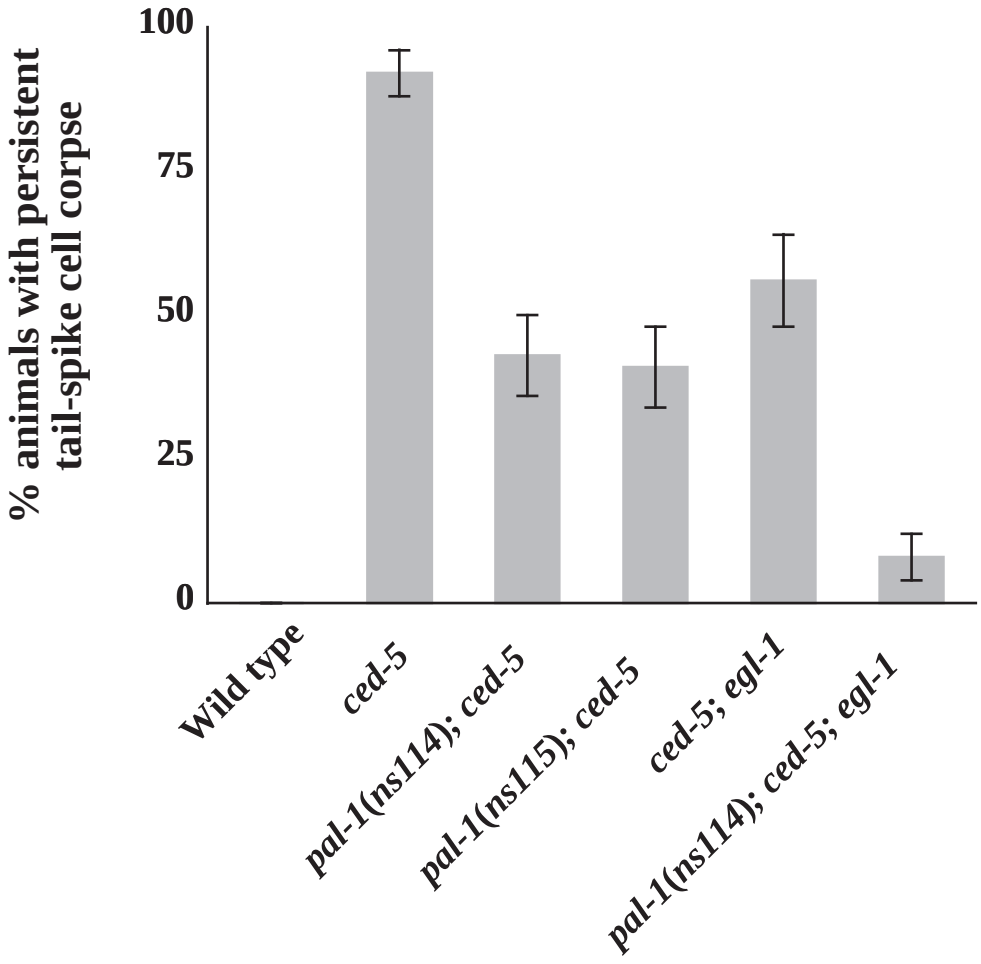

Figure 4.2: *pal-1* is required for tail-spike cell death and acts in parallel to *egl-1*. For each genotype, between 20 and 52 L1 animals were scored for persistent tail-spike cell corpses, as identified by position, shape, and expression of the *nsIs25 C. briggsae ced-3*promoter::GFP transgene. Error: standard error of the mean. Alleles used: *ced-5*(*n1812*); *pal-1*(*ns114*, *ns115*); *egl-1*(*n1084n3082*).

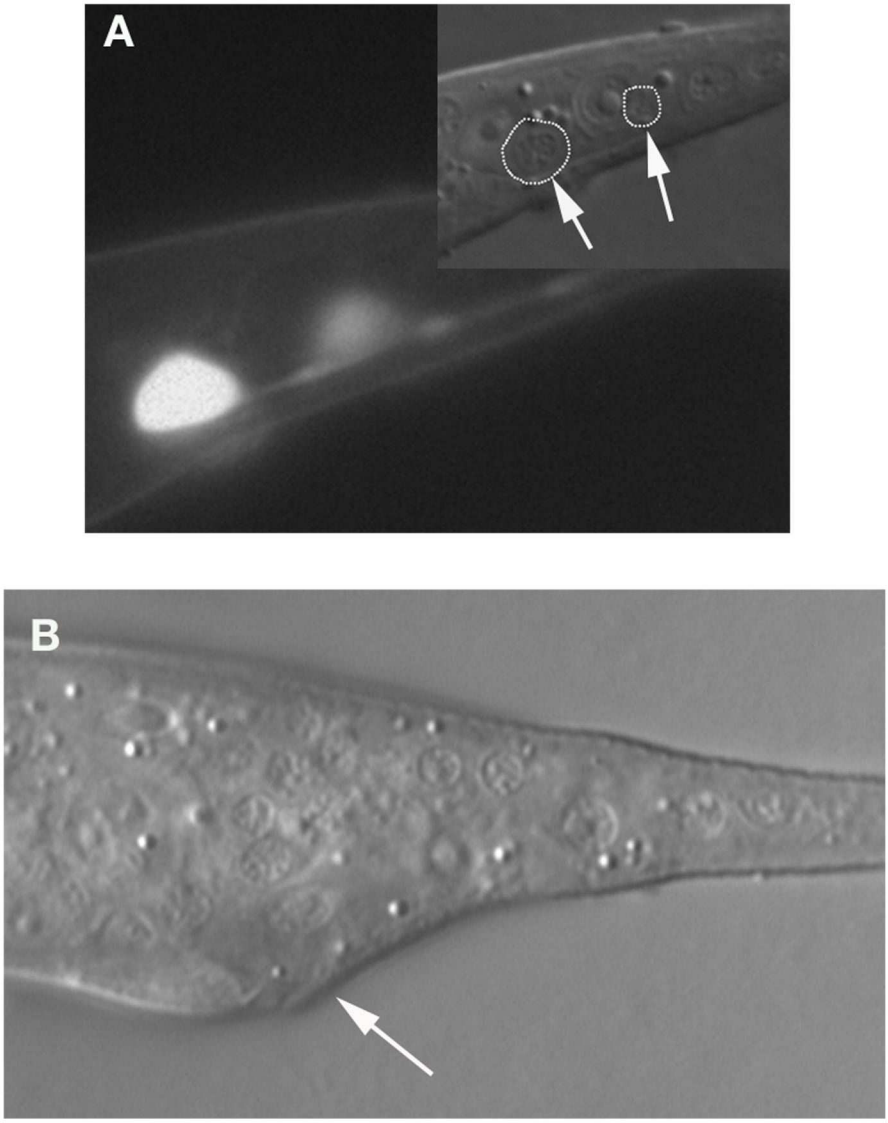

Figure 4.3: *pal-1*(*ns114*) animals possess defects in tail-spike cell fusion and tail morphology. (A) Epifluorescence and Nomarki (inset) images of the tail of a *pal-1*(*ns114*) animal carrying a 0.7 kb *C. briggsae ced-3* promoter::GFP reporter transgene (*nsIs25*). Note the presence of two mononucleate tail-spike cells (arrows) in this L2 animal. (B) Epifluorescence image of the tail of a *pal-1*(*ns114*) L1 animal. Note abnormal bulge (arrow). This defect was much less prevalent in older animals, suggesting that it may be corrected during larval growth.

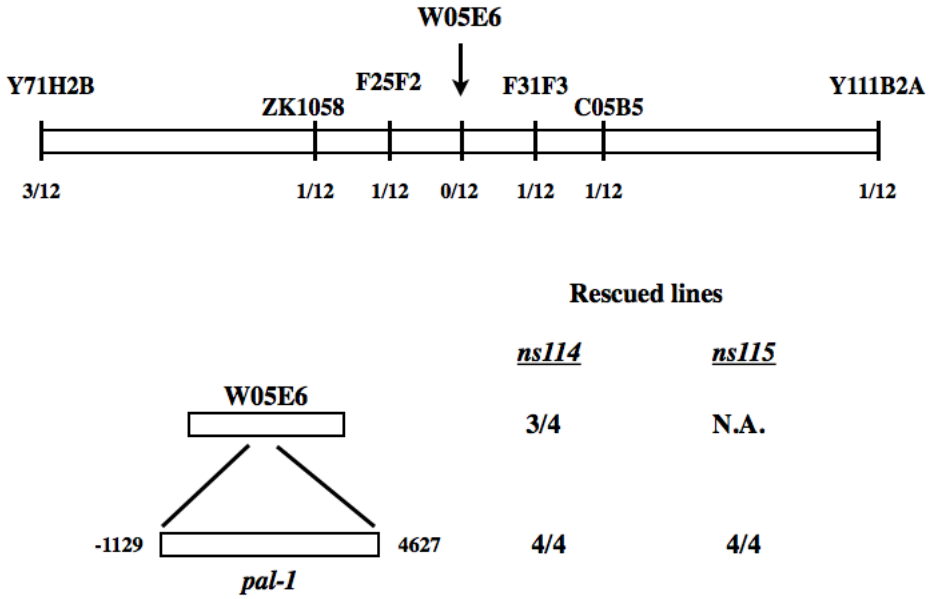

Figure 4.4: Mapping and rescue of *ns114*. Top: Schematic illustrating the approach taken to map *ns114*. *ns114*/CB4856 heterozygotes were allowed to self-fertilize, and homozogous *ns114* progeny were tested for the presence of CB4856 single nucleotide polymorphisms (SNPs). SNP locations are indicated by cosmid names above the rectangle depicting chromosome III, as is the location of the cosmid containing *pal-1* (W05E6). The number of chromosomes containing CB4856-specific SNPs out of the total examined is indicated under each SNP. Bottom: Results of rescue experiments. *pal-1*(*ns114* or *ns115*); *ced-3*(*n717*); *nsIs25* mutants were injected with the indicated rescue constructs, and animals were assessed for rescue of the defect in *ced-3* reporter expression. The number of rescued lines out of the total number of transgenic lines examined is indicated. Details of the rescue experiment are described in the text and in Chapter 8.

ns114 animals exhibited defects in tail-spike cell fusion (Figure 4.3A) and some possessed a mild tail deformity (Figure 4.3B). Tail-spike cell death defects could consistently be seen in animals displaying neither cell fusion nor tail morphology abnormalities, suggesting that the defects are independent of one another. To avoid errors in cell identification, inappropriate tail-spike cell survival was only scored in animals with an otherwise wild-type tail morphology.

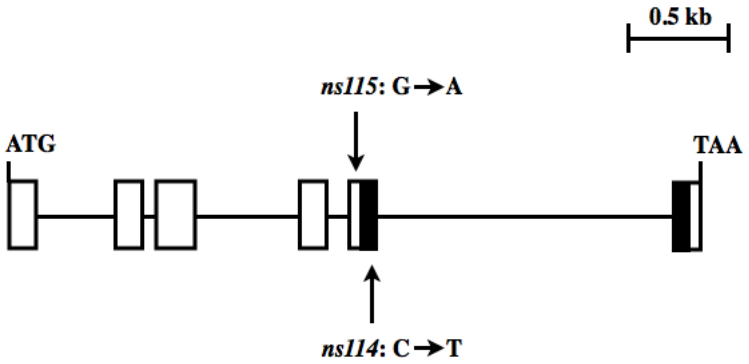

Figure 4.5: Schematic illustrating gene structure of *pal-1* and location of the *ns114* and *ns115* lesions. Boxes indicate exons, lines indicate introns, darkened boxed region is the homeodomain-encoding region.

WT	**...GCAAGT...**
ns114	**...GTAAGT...**
Splice donor consensus	**...GTAAGT...**

Figure 4.6: *ns114* results in the creation of a splice donor site within exon 5 of the *pal-1* coding sequence. Comparison of the wild-type and *ns114* mutant sites to the consensus *C. elegans* splice donor sequence; boxed region highlights the mutation.

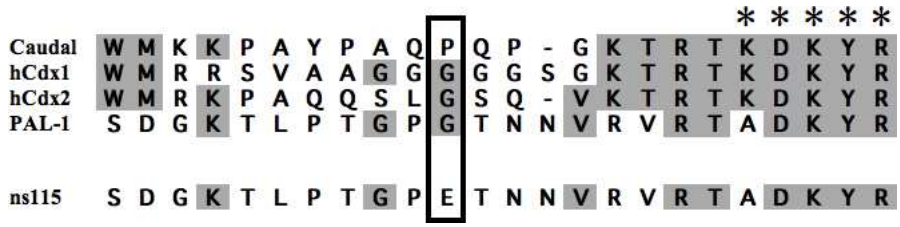

Figure 4.7: Alignment between PAL-1 and its *Drosophila* and human homologs, Caudal and Cdx1/2. Conserved residues are shaded; asterisks indicate residues comprising the homeodomain; residue mutated by the *ns115* mutation is boxed.

PAL-1 controls expression of *ced-3* in the tail-spike cell

We used single nucleotide polymorphism differences between the *ns114* and CB4856 *C. elegans* strains (Wicks et al., 2001) to map *ns114* to a 4.5 map unit interval on chromosome III containing the homeodomain transcription factor *pal-1* (Figure 4.4). Previously isolated *pal-1* mutants display tail morphogenesis defects (Edgar et al., 2001), suggesting that *ns114* might be an allele of this gene. Indeed, we found that both a 6 kb *pal-1* genomic DNA fragment and W05E6, the cosmid containing *pal-1* (Edgar et al., 2001), fully rescued the *ns114* tail-spike cell GFP expression defect (Figure 4.4). Consistent with these results, we identified a C to T alteration at position 1944 of the *pal-1* genomic sequence in *ns114* animals (Figure 4.5). This mutation creates an ectopic consensus splice-donor site (Figure 4.6) that, if used, would result in a frame shift after codon 234, altering the C-terminal region of the homeodomain, including the residues proposed to make up the DNA-binding domain (Mlodzik et al., 1985).

Like *ns114* mutant animals, *ns115* mutants exhibited weak (74%, n=51) or absent (24%, n=51) expression of the *C. elegans ced-3*promoter::GFP reporter, and a partially penetrant defect in tail-spike cell death (Figure 4.2). We mapped *ns115*, and identified a G to A missense mutation at position 1655 of the *pal-1* genomic sequence

(Figure 4.5) in this mutant. This mutation results in a glycine to glutamic acid substitution, altering a conserved residue located just outside the PAL-1 homeodomain (Figure 4.7). *pal-1* null alleles result in fully penetrant lethality accompanied by severe defects in posterior patterning (Edgar et al., 2001). Additionally, we never detected *ced-3* expression in the posterior regions of *pal-1*(*ok690*) null mutants (0%, n=30). The comparably mild defects observed in *ns114* and *ns115* mutants suggest that these are weak, or cell-specific alleles of *pal-1*, and that the ectopic splice donor site in *ns114* animals may be used only occasionally.

To learn more about *pal-1* expression in the tail-spike cell, we examined the effects of the *pal-1*(*e2091*) mutation. *pal-1*(*e2091*) mutant animals contain a mutation within the fifth intron of *pal-1* (Zhang and Emmons, 2000); this mutation results in patterning abnormalities in the male tail (Waring and Kenyon, 1990). Consistent with the requirement for this site, the *pal-1* rescue construct pSC16, which contains most of the *pal-1* genomic sequence but lacks the fifth intron, is unable to rescue the patterning defects observed in *e2091* mutant animals (Edgar et al., 2001). We found that the *e2091* mutation did not affect tail-spike cell GFP expression or survival (n=40). The pSC16 rescue construct, however, was unable to rescue the defects in *ced-3*promoter::GFP expression and tail-spike cell morphology observed in *ns114* mutant animals (2 transgenic lines examined). Therefore, while the site mutated in *e2091* is not required for proper *pal-1* expression in the tail-spike cell, other regulatory sites situated within the fifth intron may play a key role in regulating *pal-1* expression in this cell. A construct containing *pal-1* cDNA under the control of the *pal-1* promoter was also unable to rescue the defects in *ns114* mutant animals (2 transgenic lines examined), consistent with the notion that regulatory regions within *pal-1* introns may be required for appropriate expression of *pal-1*.

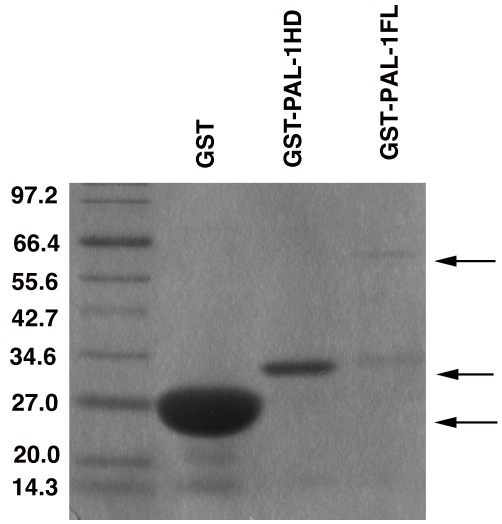

Figure 4.8: GST-PAL-1 fusion proteins, as visualized on a 10% SDS-polyacrylamide gel. Proteins ran at their expected sizes (GST: 27.8 kD, GST-PAL-1 HD (homeodomain): 34.8 kD, GST-PAL-1 FL (full-length): 56.7 kD). Broad range ladder (NEB) was used as a size marker; mass (in kD) of protein bands is indicated on the left. Arrows point to position of fusion proteins.

PAL-1 can bind *ced-3* promoter sequences

pal-1 is expressed in a number of posterior cells, including the tail-spike cell (Edgar et al., 2001), and functions cell-autonomously in the V6 cell (Waring et al., 1992) and in cells of the C and D lineages (Edgar et al., 2001). These results suggested to us that PAL-1 protein may directly bind to the *ced-3* promoter to allow *ced-3* expression in the tail-spike cell. While the consensus DNA binding site of *pal-1* has not been defined, the similarities between the homeodomains of PAL-1 and its closest *Drosophila* and vertebrate homologs, caudal and Cdx1/2, respectively, suggested to us that the proteins might share similar DNA binding affinities. Intriguingly, the caudal consensus DNA binding site, TTTAT(G) (Dearolf et al., 1989), appears in two of the three *ced-3* promoter sites we established as critical for tail-spike cell *ced-3* expression and death (sites B and C; see Chapter 3). We therefore tested the ability of PAL-1 to bind these promoter sites in an electrophoretic mobility shift assay. We

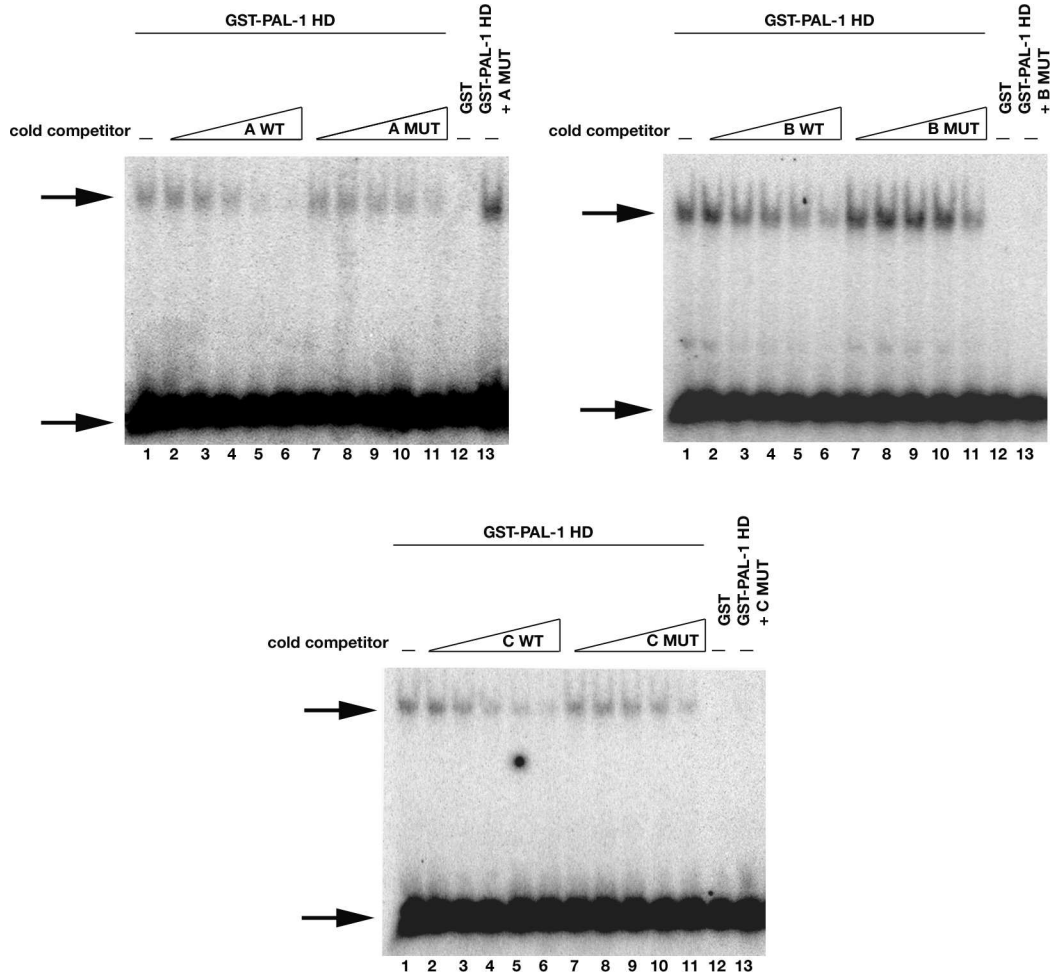

Figure 4.9: Electrophoretic mobility shift assay showing binding of PAL-1 HD to labeled DNA derived from sites A (top), B (middle) and C (bottom) of the conserved *C. elegans ced-3* promoter. Note that PAL-1 binds to sites B and C, but only exhibits non-specific binding to DNA derived from site A. Cold competitor DNA was added at concentrations of 10X (lanes 2, 7), 100X (lanes 3, 8), 200X (lanes 4, 9), 500X (lanes 5, 10) and 1000X (lanes 6, 11), as indicated by triangles above the autoradiogram. Lane 12 shows no binding of GST to labeled A, B or C DNA; lane 13 shows binding of PAL-1 HD to labeled mutant oligonucleotide. Positions of shifted PAL-1 homeodomain-DNA complex and unbound DNA are indicated with arrows. Details of the assay can be found in Chapter 8.

expressed fusion proteins between glutathione S-transferase (GST) and the PAL-1 homeodomain (residues 203-270), or the PAL-1 full-length protein (Figure 4.8). As shown in Figure 4.9, the GST-PAL-1 homeodomain fusion protein bound ^{32}P-labelled 22 bp oligonucleotides from the *ced-3* promoter containing either sites B or C. Binding with the PAL-1 full-length protein was not tested. Binding was competed by cold wild-type oligonucleotides, but was less efficiently competed by mutant B or C oligonucleotides in which the consensus binding site was mutated to TGGAT. Similar results were obtained using oligonucleotides in which all five consensus binding residues were altered to ACGCC (data not shown). These results demonstrate that PAL-1 is able to bind sites within regions B and C in a sequence-specific manner. Consistent with this conclusion, the PAL-1 fusion protein did not bind ^{32}P-labelled mutant B or C oligonucleotides (Figure 4.9). Additionally, when identical mutations were made in the *C. elegans ced-3* promoter::GFP reporter, expression in the tail-spike cell was greatly compromised. For example, in 4 lines containing the B site mutation, 4/100 animals examined had weak GFP expression and 96/100 had no GFP expression in the tail-spike cell. Similarly, in 4 lines containing the C site mutation, 74/120 animals examined had weak GFP expression and 28/120 had no expression in the tail-spike cell. Mutating both the B and C sites completely abrogated reporter expression in the tail-spike cell (6/6 lines).

Taken together, these results support the idea that PAL-1 protein binds *ced-3* promoter sequences in the tail-spike cell to promote cell death. We were unable to detect sequence-specific binding of PAL-1 to the third *ced-3* promoter site required for tail-spike *ced-3* expression and cell death (site A; Figure 4.9), suggesting that this site may bind yet another factor required for induction of *ced-3* expression in the tail-spike cell.

Based on our model, we predict that *pal-1* acts cell-autonomously in the tail-spike cell to directly promote *ced-3* expression. This is a reasonable assumption given that

pal-1 is a transcription factor, and we have shown that *pal-1* can directly bind to critical sites within the *ced-3* promoter required for the gene's expression in the tail-spike cell. In addition, previous reports have established that *pal-1* is expressed in the tail-spike cell (Edgar et al., 2001). In *C. elegans*, mosaic experiments are often performed to establish in which cells a given gene is required. Animals genotypically mutant for a given gene are transformed with a rescue transgene carrying a wild-type copy of this gene. During mitosis, spontaneous loss of this rescue transgene occurs at low frequency in some cells, resulting in animals that are genotypically wild-type in some cells and genotypically mutant in others. In our case, however, this was not a feasible means for determining whether *pa1-1* acts cell-autonomously in the tail-spike cell. The tail-spike cell is born as two cells that are "mirror images" of one another in the *C. elegans* lineage. Both cells are derived from the first posterior daughter cell in the AB lineage; after this point, the two cells diverge in the lineage, ending up as left and right equivalents after a long line of cell divisions (AB.p(l/r)ppppppa). Each of these cells is born as the sister of a hyp10 cell. In addition to being closely related to the tail-spike cells, the hyp10 cells are also located adjacent to the tail-spike cells in the developing embryo (Sulston et al., 1983). To determine whether *pal-1* activity in the tail-spike cell is sufficient to promote *ced-3* expression in this cell by mosaic analysis, the *pa1-1* rescue transgene would need to be lost in both hyp10 cells. The low probability of this event precludes this as a viable approach. As an alternate approach, we attempted to drive *pal-1* cDNA in the tail-spike cell using a promoter specific to the tail-spike cell. While our *C. elegans ced-3*promoter::GFP reporter is expressed broadly in the anterior embryo (data not shown), expression in the tail appears to be, for the most part, specific to the tail-spike cell (see Figure 3.2). We therefore expressed *pal-1* cDNA under the control of this promoter, and asked whether the construct was able to rescue the defects in tail-spike cell morphology and *ced-3* expression observed in *ns114* mutants. We did not observe rescue in any of the

five transgenic lines we examined. This result may be consistent with the notion that *pal-1* does not act cell-autonomously to promote *ced-3* expression in the tail-spike cell. However, our results are difficult to interpret given that expression from the *ced-3* promoter is induced only shortly before the tail-spike cell dies (see Chapter 3). It is possible, and indeed quite likely, that *pal-1* expression may be required earlier in order to promote tail-spike cell fusion and *ced-3* expression.

Pax-2 homologs are not required for tail-spike cell death

In addition to the non-biased screen described above, we also took a bioinformatics approach to identify regulators of *ced-3* expression. Using the MatInspector software, we searched for transcription factors whose binding sites were found within the region of the *ced-3* promoter containing sites A, B and C (-1210 to -1125). The paired domain-containing transcription factor Pax-2 emerged from this search. Two Pax-2 homologs have been identified in *C. elegans*, *pax-2* and *egl-38*. Interestingly, *egl-38* is expressed in the male and hermaphrodite tail, where it is required for posterior patterning (Chamberlin et al., 1997). *pax-2* and *egl-1* have also recently been demonstrated to play a role in cell survival by directly regulating *ced-9* transcription (Park et al., 2006). We tested the role of these genes in tail-spike cell *ced-3* expression and tail-spike cell death, and found that *ced-3* promoter::GFP expression was unaltered by the loss-of-function mutations *pax-2*(*ok935*) and *egl-38*(*sy294*) (data not shown). Tail-spike cell death was also unaffected (number of animals with inappropriately surviving tail-spike cells: *pax-2*(*ok935*): 0/13; *egl-38*(*sy294*): 0/14). We did not test whether the tail-spike cell dies prematurely in these mutant backgrounds.

4.3 Conclusions

We have demonstrated that *ced-3* expression is induced in the tail-spike cell shortly before the cell meets its demise, suggesting that *ced-3* transcriptional upregulation

may play a critical role in initiating death of the tail-spike cell. Here, we discuss the results of a genetic screen designed to isolate regulators of this expression. Most notably, we identified the homeodomain-containing transcription factor *pal-1* as a critical mediator of *ced-3* expression in the tail-spike cell. We show that *pal-1* is also required for tail-spike cell death, thereby implying that death of this cell is dependent upon upregulation of *ced-3* transcription. Using *in vitro* studies, we have demonstrated that PAL-1 is able to bind the *ced-3* promoter at sites that are critical for both of these processes, suggesting that *pal-1* directly promotes *ced-3* expression. Our results unveil a novel mechanism for controlling the timing of programmed cell death by transcriptional regulation of caspases.

Chapter 5

Is tail-spike cell death dependent on the completion of upstream cellular events?

5.1 Background

The tail-spike cell exhibits a unique and elaborate developmental program. Born embryonically roughly 280 minutes post-cleavage, the pair of tail-spike cells fuses and extends a posterior filamentous process before meeting its demise at 600 minutes, over five hours after being born (Sulston et al., 1983). Both cell fusion and process extension can occur independently of an intact cell death machinery, as tail-spike cell morphology is unaffected by defects in the general cell death machinery (see Chapter 2). We wished to test the converse: is tail-spike cell death dependent upon these temporally upstream events? We hypothesize that programmed cell death may function as a read-out for proper execution of tail-spike cell development, and that mutants in upstream developmental processes may exhibit inappropriate tail-spike cell survival. For example, fusion of the tail-spike cells or extension of the cell's posterior process might affect temporally downstream PCD by subsequent reprogramming of cell fate or by altering cellular contacts. While the factors governing tail-spike cell fusion and process extension are as of yet unknown, several factors involved in these processes in other cells have been characterized. We tested whether these factors play a role in tail-spike cell morphogenesis and tail-spike cell death.

5.2 Results

Tail-spike cell fusion and tail-spike cell death

To test whether tail-spike cell fusion is a necessary precursor for tail-spike cell death, we examined the effects of *eff-1* loss-of-function mutations. *eff-1* encodes a type I transmembrane protein that is expressed in fusing cells shortly before their fusion and is required for all epidermal cell fusion in *C. elegans* (Mohler et al., 2002). Surprisingly, neither of the *eff-1*(lf) alleles we examined blocked tail-spike cell fusion. Tail-spike cell *ced-3*promoter::GFP expression and tail-spike cell death were also unaffected by *eff-1* mutations (Table 5.1). Previous reports have indicated that *eff-1*(lf) mutations do block the fusion of tail hypodermal cells adjacent to the tail-spike cell (Mohler et al., 2002), suggesting that tail-spike cell fusion and death can occur independently of the fusion of the cell's neighbors.

We found that a loss of function mutation in *lin-44*, one of five *C. elegans* Wnt ligands, weakly blocked tail-spike cell fusion (11%, n=38). Despite this fusion defect, *ced-3*promoter::GFP expression was unaltered in *lin-44*(*n1792*) mutant animals (Table 5.2), suggesting that fusion and *ced-3* expression may be independent processes in the tail-spike cell. *pal-1*(*ns114*) and *pal-1*(*ns115*) mutant animals are also partially defective in tail-spike cell fusion (see Chapter 4). We observed that, in *pal-1*(*ns114*) mutant animals, a uni-nucleate tail-spike cell can die independently of fusion with its partner cell. Specifically, we found that 11% (n=46) of *pal-1*(*ns114*); *ced-5*(*n2812*) L1 stage double mutant animals possessed both an inappropriately surviving tail-spike cell and a tail-spike cell corpse. However, though the tail-spike cell can clearly die without fusing, our findings do not preclude the possibility that fusion may nonetheless play a weak role in promoting tail-spike cell death.

Table 5.1: *eff-1* is not required for tail-spike cell fusion or for tail-spike cell death.

Genotype[a]	% fused tail-spike cells[b]	% tail-spike cells expressing *ced-3*::GFP[b]	% surviving tail-spike cells[b]
eff-1(hy40)	97 ± 3[c]	94 ± 4[c]	3 ± 3[c]
eff-1(hy21ts)[d]	90 ± 6	100	3 ± 3

[a]All strains contained the *nsIs25 C. briggsae ced-3* promoter::GFP integrated transgene for scoring tail-spike cell survival and tail-spike cell fusion. [b]Between 30 and 40 L3 stage *ced-3(n717)* animals were scored for fusion defects and abnormalities in *ced-3*promoter::GFP expression. Survival was scored in at least 30 L1 stage animals in an otherwise wild-type background. [c]Standard error of the mean. [d]Worms were propagated at 25 °C.

Table 5.2: Tail-spike cell process extension and tail-spike cell death are unaffected by loss-of-function mutations in many of the genes involved in process outgrowth and directional pathfinding in other cells.

Genotype[a]	% tail-spike cells with wild-type posterior process[b] (n)	% tail-spike cells expressing *ced-3*::GFP[b] (n)	% surviving tail-spike cells[b] (n)
Wild-type	100	100	0
lin-17(n671)	100	100	3 ± 3^c
lin-44(n1792)	100	97 ± 3^c	NA
vab-8(e1017)	100	100	0
unc-53(e404)	94 ± 6^c	94 ± 6	0
unc-73(e936)	100	100	0
unc-76(e911)	98 ± 2	100	0
unc-5(e53)	95 ± 5	96 ± 4	0
unc-40(e271)[d]	NA	NA	0

[a]All strains contained either the *nsIs23* or the *nsIs25 C. briggsae ced-3* promoter::GFP integrated transgene for scoring tail-spike cell survival and tail-spike cell process extension. [b]Process extension and *ced-3*promoter::GFP expression were scored in at least 20 L1-L2 stage *ced-3(n717)* or *ced-4(n1162)* animals. Survival was scored in at least 20 L1-L2 stage animals in an otherwise wild-type background. [c]Standard error of the mean. [d]Strain also contained *dpy-5(e61)*. NA, not assessed.

Factors required for extension of the tail-spike cell's posterior process remain unknown

To ascertain the role of process extension in tail-spike cell death, we first needed to identify genes required for formation of the tail-spike cell's posterior process. We examined the role of several genes that are required for process extension in other cells, and that are expressed in the posterior region of the worm. Specifically, we tested the requirement for each of the following genes: *vab-8*, which encodes a protein containing an atypical kinesin-like motor domain and is involved in axon outgrowth and posteriorly directed cell migration (Wolf et al., 1998); *unc-53*, a gene involved in anteroposterior outgrowth (Stringham et al., 2002); *unc-73*, which encodes a guanine nucleotide exchange factor and is required for cell migration and axonal outgrowth (Steven et al., 1998); *unc-76*, which is required for axonal outgrowth (Bloom and Horvitz, 1997); and *unc-5*, a netrin receptor encoding-gene that provides instructive cues to guide cellular migration (Hamelin et al., 1993). Using our tail-spike cell reporter, we examined tail-spike cell morphology in *vab-8*, *unc-53*, *unc-73*, *unc-76* and *unc-5* loss-of-function mutant backgrounds. Tail-spike cell morphology was not altered by loss-of-function mutations in any of these genes; tail-spike cell *ced-3* reporter expression and tail-spike cell death were also unaffected (Table 5.2). Additionally, tail-spike cell death was not affected by a loss-of-function mutation in *unc-40*/netrin receptor/DCC (Table 5.2).

lin-17 and *lin-44*, *C. elegans* homologs of the frizzed receptor and Wnt ligand, respectively, are expressed in tail hypodermal cells and have been shown to play a role in neuronal polarity (Hilliard and Bargmann, 2006, Pan et al., 2006). Loss-of-function mutations in these genes, however, had no effect on polarity or extension of the tail-spike cell process, nor did they affect tail-spike cell death (Table 5.2). Intriguingly, polarity of the tail-spike cell process was altered in *eff-1*(*hy21ts*) mutant animals. Specifically, the tail-spike cell process extended anteriorly rather than posteriorly in

27% of *eff-1*(*hy21ts*); *ced-3*(*n717*) L1 stage animals (n=30). As reported above, tail-spike cell fusion and tail-spike cell death are unaffected by *eff-1* loss-of-function mutations. The defect in process extension may instead result from the failure of neighboring cells to fuse, and the absence of non-cell-autonomous cues required for positional information.

At this point, the role of process extension in tail-spike cell death remains unclear. The candidate genes we examined are by no means an exhaustive collection of guidance factors; it is also possible that some or all of these genes act in a redundant manner to direct tail-spike cell process formation. Hopefully, future studies will shed light on the role of tail-spike cell process extension, and, more generally, on the role of upstream morphological events, on the initiation of tail-spike cell death.

Loss-of-function mutations in *ced-2*, *ced-5* and *ced-10* do not affect position of the tail-spike cell

Engulfment of a dying corpse is dependent upon cytoskeletal rearrangements within the engulfing cell. Accordingly, several of the genes required for engulfment also mediate various other cellular processes. Specifically, *ced-2*/CrkII, *ced-5*/DOCK 180, *ced-10*/Rac and *ced-12*/ELMO1 are required during larval development for proper migration of the gonadal distal tip cells (Wu and Horvitz, 1998; Reddien and Horvitz, 2000; Gumienny et al., 2001; Wu et al., 2001). We did not observe defects in tail-spike cell morphology or position in animals carrying loss-of-function mutations in these genes (data not shown).

Screen for mutants defective in tail-spike cell death

In addition to the candidate approach described above, we also took a non-biased approach intended to identify signaling factors that when mutant result in inappropriate

tail-spike cell survival. We performed a pilot screen in which F2 progeny of mutagenized *nsIs23* and *nsIs25* animals (rounds one and two of screening, respectively) were assayed as early larvae for inappropriate tail-spike cell survival as determined by GFP reporter expression. We expected to pick up worms with defects in the already characterized general cell death machinery as well as those with defects in novel factors, including putative upstream signaling factors mediating the tail-spike cell's decision to die. We screened through 3,000 haploid genomes and identified eight independent mutants with an inappropriately surviving tail-spike cell (Table 5.3). The tail-spike cell persisted until adulthood in all eight of these mutants, suggesting that cell death is being prevented and not merely delayed in these animals. Preliminary characterization of these mutants is described below.

Table 5.3: Mutants isolated in a screen designed to detect genes involved in tail-spike cell death.

Genotype[a]	% surviving tail-spike cells[b]
Wild-type	0
ns16	60 ± 8[c]
ns17	35 ± 8
ns18	52 ± 8
ns19	25 ± 8
ns38	100 ± 0
ns39	95 ± 5
ns40	11 ± 11
ns41	17 ± 7

[a]All strains contained either the *nsIs23* or the *nsIs25 C. briggsae ced-3* promoter::GFP integrated transgene. [b]Between 10 and 40 L1 stage animals were scored for inappropriate survival of the tail-spike cell as determined by GFP reporter expression. Defects in pharyngeal cell death were not quantified. [c]Standard error of the mean.

We hypothesized that the upstream signaling factors regulating tail-spike cell death might also specify tail-spike cell fate in a broader sense, and examined our mutants for defects in tail or tail-spike cell morphology. Notably, we observed variable positioning of the tail-spike cell in *ns17* mutant animals (data not shown), suggestive of a defect in a factor involved in short-range migration. Inappropriate survival of the tail-spike cell in this mutant may point to a role for cell-cell interaction in induction of tail spike cell death. In addition, defects in tail-spike cell fusion were observed in *ns18* and *ns19* mutant animals (data not shown), possibly suggesting that tail-spike cell death may be partially dependent upon tail-spike cell fusion. Alternatively, it is possible that *ns18* and *ns19* worms lack an inductive signal required for both fusion-competence and cell death. The tails of *ns18* and *ns40* mutant animals were also grossly abnormal (data not shown), suggestive of defects in patterning of the tail. Backcrosses should be performed in order to determine whether these defects result from the same mutation that is contributing to inappropriate tail spike cell survival.

The design of the screen did not restrict our findings to genes mediating tail-spike specific cell death. Indeed, animals from five of the mutants we isolated, *ns16*, *ns18*, *ns38*, *ns40* and *ns41*, also possessed inappropriately surviving cells in the anterior pharynx, suggestive of a more general cell death defect. The presence of extra cells was not carefully quantified, and we therefore cannot rule out that *ns17*, *ns19* and *ns39* may also possess weak defects in the general cell death machinery. The pharyngeal cell death defect should be quantified in all mutants, and complementation analysis using loss of function alleles of *ced-3*, *ced-4*, and *egl-1* should be performed.

5.3 Conclusions

We hypothesized that the tail-spike cell's decision to die may be dependent upon factors signaling completion of temporally upstream cellular events. We took a candidate gene approach to identify genes required for cellular events that would be

easily identifiable using our tail-spike cell reporter, namely tail-spike cell fusion and process extension. Unfortunately, loss-of-function mutations in the candidate genes we examined affected neither tail-spike cell morphology nor tail-spike cell death. We did note that the *pal-1* gene, which we had isolated in a screen for regulators of *ced-3* expression in the tail-spike cell, is required for tail-spike cell fusion. In *pal-1* mutant animals, tail-spike cell death can occur in the absence of cell fusion, indicating that these may be independent processes. However, our results are inconclusive, as we cannot rule out that fusion plays a partial role in promoting cell death. At this point, the upstream events signaling tail-spike cell death should be further pursued. In particular, the mutants isolated in the screen described above should be characterized, and further rounds of screening should be undertaken. While we have focused our energy on better understanding tail-spike cell fusion and process extension, additional upstream events, both cell-intrinsic and cell-extrinsic, may play a role in tail-spike cell death, and should also be considered.

Chapter 6

Discussion

A model for the control of tail-spike cell death in *C. elegans*

Tail-spike cell death exhibits two salient features. First, we have shown here that the *egl-1* and *ced-9* genes, which are required for the majority of somatic cell deaths in *C. elegans*, only play a minor role in the demise of the tail-spike cell, suggesting that another pathway must exist that specifically regulates the death of this cell. Second, we have shown that, in the tail-spike cell, *ced-3* caspase is expressed minutes before the cell displays obvious signs of death, suggesting that regulation of tail-spike cell death may be achieved through transcriptional control of *ced-3*. Indeed, mutations in the *pal-1* homeodomain gene, which promotes *ced-3* transcription, specifically prevent tail-spike cell death. Taken together, these observations suggest the following model: in the tail-spike cell, *egl-1* and *ced-9* may have attenuated function, thus allowing CED-4 protein to remain unchecked. In the absence of any CED-3 caspase, CED-4 is unable to promote cell death, thus allowing the tail-spike cell to live. Upon transcription of *ced-3*, accumulating CED-3 protein may become immediately processed, through interactions with CED-4, leading to rapid killing of the cell (Figure 6.1). An important feature of this model is that killing of the tail-spike cell by CED-3 does not occur by mere overexpression of this caspase, since *ced-4* is still required for tail-spike cell death. Thus, although overexpression of CED-3 can kill cells in a CED-4-independent manner (Shaham and Horvitz, 1996a), this is not the case in the

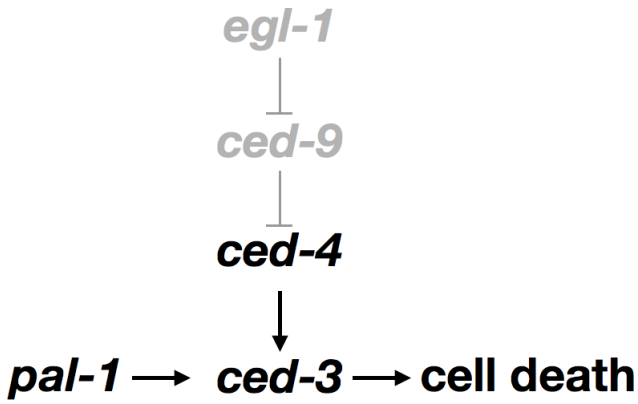

Figure 6.1: Model for regulation of tail-spike cell death. EGL-1 and CED-9 (gray) play a minor role in regulating tail-spike cell death, whereas PAL-1-mediated transcription of *ced-3* is a key regulatory module.

tail-spike cell.

A number of observations support the model described here. For example, the model predicts that mutations in *pal-1* should affect tail-spike cell death independently of mutations in *ced-9*. Indeed *pal-1*(lf); *ced-9*(lf); *ced-3*(lf) mutants have significantly more tail-spike cell survival than *ced-9*(lf); *ced-3*(lf) animals. Similarly, *pal-1* functions independently of *egl-1*. In addition, we have examined an *egl-1*promoter::GFP reporter transgene in the tail-spike cell, and have only been able to detect very low levels of expression as compared with other cells expressing the reporter (data not shown), consistent with the idea that *egl-1* plays a minor role in tail-spike cell death.

Although our data clearly demonstrate transcriptional control of *ced-3* during tail-spike cell death, *ced-4* transcription may either be constitutive or induced in this cell. We have been unable to examine *ced-4* expression in the tail-spike cell using currently available reagents, and thus cannot distinguish between these two possibilities. Regardless of whether *ced-4* is also transcriptionally upregulated in the tail-spike cell, it is evident that regulation of death of this cell must still utilize an *egl-1*/*ced-9*-independent pathway, making the tail-spike cell an exciting venue in which

to study *egl-1*/*ced-9*-independent cell death.

Our studies describe a role for PAL-1 protein in controlling *ced-3* expression in the tail-spike cell. However, PAL-1 is unlikely to be acting alone in this cell to promote *ced-3* transcription. PAL-1 is expressed in many cells in the animal that do not die (Edgar et al., 2001). Furthermore, within the tail spike cell, PAL-1 is expressed several hours before tail-spike cell death occurs. Thus, PAL-1 must either associate with other factors that promote *ced-3* transcription, or must be post-translationally activated to induce *ced-3* transcription. It is interesting to note that we could not detect specific binding of PAL-1 to the A site of the *ced-3* promoter, suggesting that this site may indeed be occupied by another protein that functions together with PAL-1 to induce *ced-3* transcription.

Our studies suggest that control of caspase transcription may be an important mechanism for exercising temporal control of cell death initiation in other animals as well. In this context, it is worth noting that caspase expression during development of any organism has not been extensively studied.

Control of cell death timing in other *C. elegans* cells

Although the *egl-1* gene is required for the death of most somatic cells destined to die in *C. elegans*, it is clear that additional mechanisms must exist that control the onset of these deaths. Specifically, some *ced-9*(lf); *ced-3*(weak lf) double mutants contain the same complement of cells as wild-type animals (Hengartner and Horvitz, 1994), suggesting that cells that normally die during *C. elegans* somatic development can die in the absence of *ced-9*, and by extension, in the absence of *egl-1*. How might cell death timing be controlled in these cells? Our examination of the pattern of expression of *ced-3* in *C. elegans* suggests that transcriptional control of *ced-3* is unlikely to be the main mechanism in these cells. Although *ced-3* expression can be seen in some dying cells during embryogenesis, most expression is confined to cells

that live (data not shown), suggesting that *ced-3* may be expressed in precursors of cells destined to die. Thus, asymmetric segregation, or activation of CED-3 protein (or mRNA) during cell division may be important for other cell deaths in *C. elegans*. Intriguingly, most cells that die in the *C. elegans* soma do so within 30-60 minutes after being born. This time interval is of the same order as the gap between the onset of *ced-3* transcription and the first signs of death in the tail-spike cell. This observation suggests that in other *C. elegans* cells, asymmetric segregation of CED-3 protein (or mRNA) may function in a similar manner to transcriptional upregulation of *ced-3* in the tail-spike cell.

The *ced-9*(*n1950*) gain of function allele may not only block CED-9/EGL-1 association

Our studies have demonstrated that *egl-1* and *ced-9* behave differently in the tail-spike cell as compared to other cells destined to die in two ways. First, we found that *egl-1*(lf) mutations block tail-spike cell death in 30% of animals examined, whereas the *ced-9*(*n1950*gf) mutation does not block tail-spike cell death. In all other somatic or germ cell deaths that have been examined, *ced-9*(*n1950*gf) and *egl-1*(lf) mutants have exhibited identical phenotypes (Conradt and Horvitz, 1998; Gumienny et al., 1999; Gartner et al., 2000). Second, in the tail-spike cell *ced-9* lacks its death-promoting function. Specifically, *ced-9* has been shown to have both death-preventing as well as death-promoting functions in many somatic cells (Hengartner and Horvitz, 1994). The nature of the death-promoting function of *ced-9* is not understood; however, genetic interaction studies suggest that *ced-9* has the capacity to inhibit two alternatively spliced *ced-4* transcripts: *ced-4*S, which promotes cell death, and *ced-4*L, which inhibits cell death (Shaham and Horvitz, 1996b). Inhibition of the former by *ced-9* could explain the death-preventing function of *ced-9*, and inhibition of the latter by *ced-9* could explain the death-promoting function of *ced-9* (Shaham and

Horvitz, 1996b).

How might the disparities between *egl-1* and *ced-9* functions in the tail-spike cell be resolved? Genetic and structural studies suggest that the *ced-9*(*n1950*gf) mutation may block the association of CED-9 with EGL-1 (Parrish et al., 2000; Yan et al., 2004). However, if this were the sole mechanism of *n1950* function, then *egl-1*(lf) and *ced-9*(*n1950*gf) mutants should exhibit identical phenotypes, which is not the case in the tail-spike cell. One resolution of this apparent contradiction is to suggest that the *ced-9*(*n1950*gf) mutation has two effects. First, this mutation may only partially block association of CED-9 with EGL-1. Second, *n1950* may fully block the death-promoting function of CED-9; for example, *n1950* may block the ability of CED-9 to inhibit CED-4L function. Thus, according to this hypothesis, *n1950* should behave like a weak *egl-1* mutation in the tail-spike cell, only very mildly preventing cell death, since the death-promoting function of *ced-9* is not present in this cell. In other somatic cells, *ced-9* does possess a death-promoting function (perhaps because CED-4L is expressed in these cells and not in the tail-spike cell), and thus *n1950* interferes both with that function and with binding to EGL-1, resulting in extensive cell survival. Testing this hypothesis will require a clearer understanding of the nature of the *ced-9* death-promoting function.

Caspase transcription and the control of tumorigenesis

Caspases have been demonstrated to play a role in tumorigenesis (Stupack et al., 2006), though the mechanism by which these proteases suppress tumor progression, and the factors regulating their expression, remain poorly understood. Our results suggest that mutations in transcriptional regulators of caspases may promote tumorigenesis by blocking cell death. Intriguingly, mutations in the vertebrate homolog of *pal-1*, Cdx2, promote digestive tract tumor formation (Chawengsaksophak et al., 1997; Aoki et al., 2003; Bonhomme et al., 2003), and tumor aggressiveness is inversely

correlated with levels of Cdx2 expression (Hinoi et al., 2001). Furthermore, in the intestinal epithelium, Cdx2 is expressed only at low levels in less-differentiated cells near the intestinal crypt, and at high levels in fully differentiated cells of the epithelium, which continually undergo apoptosis (Silberg et al., 2000). Taken together, these observations raise the possibility that Cdx2 promotes vertebrate caspase transcription to effect programmed cell death in the intestinal epithelium in a manner similar to *pal-1* regulation of *ced-3* expression in *C. elegans*.

Discarding an unnecessary cell

We have demonstrated that the onset of tail-spike cell death is controlled by *pal-1*-mediated upregulation of *ced-3* expression, and speculate that *pal-1* acts with other factors to specify tail-spike cell death with incredible temporal precision. However, we still have not answered several critical questions regarding the cell's death. The tail-spike cell survives for over five hours, during which time it is transformed from two single cells into a single binucleate cell with an elegant posterior process. Why does the tail-spike cell subsequently commit itself to death? How do upstream cellular events contribute to the cell's terminal differentiation step? We speculated that the tail spike cell might die as a result of upstream signaling informing the cell that it has finished what it had set out to accomplish: differentiated, and completed its as of yet unknown function. EM studies (Sulston et al., 1983) point to a role for the tail-spike cell process in formation of the streamlined *C. elegans* tail spike. Completion of this process may provide cell-intrinsic or extrinsic cues signaling the cell to die. Parallels to this model can be found throughout invertebrate and vertebrate development (reviewed in Jacobson et al., 1997 and Meier et al., 2000). During insect metamorphosis, muscles and neurons needed for larval but not for adult locomotion are lost by ecdysone-mediated transcriptional regulation of a number of apoptotic

genes (Dorstyn et al., 1999). The destruction of evolutionary remnants is also dependent upon PCD-mediated killing; interdigital cells are removed via BMP (bone morphogenic protein)-mediated induction of PCD (Zou and Niswander, 1996). We have been unsuccessful in our attempts to define the upstream cellular events required for the tail-spike cell's demise, or to better understand the cell's function; both areas should be further pursued. Defining *pal-1* co-factors in the tail-spike cell might also provide insight into how upstream cellular events trigger cellular suicide.

Why control caspases transcriptionally?

Transcriptional regulation of cell death onset has been documented in several systems. During *Drosophila* development, expression of the cell death activators *rpr* and *grim* precedes and may in fact trigger cellular demise (reviewed in Tittel and Steller, 2000; see also Chapter 1). In *C. elegans*, transcription of *egl-1* has been proposed to play a critical role in specifying the onset of cell death in several cells (Conradt and Horvitz, 1999; Thellmann et al., 2003; Liu et al., 2006; Hoeppner et al., 2004). However, as of yet, transcriptional regulation of a caspase has not been examined in great detail. In fact, quite the opposite model has been put forward: the cell death execution machinery is thought to be ubiquitously expressed, and caspase activity believed to be regulated post-translationally. In contrast, we have shown that controlling the expression of *ced-3* caspase is an important means of regulating cell death in the *C. elegans* tail-spike cell. Why regulate cell death in this manner?

Most cells fated to die in *C. elegans* meet their demise within thirty minutes after being born. Cells destined for such a short life might require that an intact cell death execution machinery be present from the moment of their birth; cells requiring rapid induction of cell death might have similar requirements. The tail-spike cell lives ten times longer than most cells destined to die in *C. elegans*. Though its function is as of yet undefined, the tail-spike cell's elaborate morphology is highly suggestive of

functional relevance. These two attributes may necessitate an alternate mechanism for activating tail-spike cell death. The small number of cells in *C. elegans* limits the level of redundancy, and it is likely that the tail-spike cell does not have a "substitute" available in the event of its absence. The cell may limit levels of *ced-3* as a means of safeguarding against premature death. The CED-3 pro-protein has weak intrinsic protease activity, and the presence of low levels of activated CED-3 might compromise the cell's ability to maintain exquisite control over the time of its death. The tail-spike cell might also be frugal, and proteolysis of CED-3 substrates over time may be undesirable from an energetic point of view. In addition, given that the time of the cell's death has been predetermined, it may not want to invest the energy to transcribe and translate a gene that will only be needed much later in life, given that translated protein degrades over time. Other irreplaceable cells whose function is critical to the organism may regulate their death via a similar mechanism.

Chapter 7

Future Directions

The tail-spike cell is thought to be involved in formation of the *C. elegans* tail, a role that has been inferred from electron micrograph studies of the embryo (Sulston et al., 1983). However, there is no direct evidence supporting this claim, and the function of the cell remains unknown. We hypothesize that the tail-spike cell's decision to die is linked to completion of its function, and therefore propose that understanding the cell's function may yield insight into its death. Tail-spike cell function may be ascertained by laser ablating the cell shortly after its birth, taking advantage of a commonly utilized method to determine cell function in *C. elegans*. Currently, there are no markers to assist in identifying the tail-spike cell in its early stages of development, and cell identification will need to be done by following the cell from its birth using Nomarski optics. While we predict the ablated worms will exhibit a defect in gross tail morphology, other defects should also be considered. The tail-spike cell may play a role in modeling not only the hermaphrodite tail but the male tail as well. Ablations should thus also be performed in a genetic background containing a *him* (high incidence of males) mutation. The tail-spike cell may also act as a guidepost for other cells extending processes into the tail, including neurons. For example, PLM(L/R), two of the six touch receptor neurons, extend processes into the tail. If these processes are disrupted, we might expect a defective response to touch in the rear of the animal; this possibility should be considered and ablated animals should

be assayed for touch responsiveness.

Depending on the ease with which the "ablation" phenotype can be visualized, it may be possible to screen for factors required for tail-spike cell fate and function by screening for worms with a phenotype similar to that of ablated worms. We would expect to isolate mutants in which tail-spike cell function was mis-specified, or in which the tail-spike cell died prematurely. Alternatively, if the "ablation" phenotype is specific enough, it may also be feasible to take a candidate gene approach to test whether mutations resulting in a similar gross phenotype also affect specification of tail-spike cell fate. Such experiments may also yield insight into factors responsible for repressing tail-spike cell death.

We hypothesize that tail-spike cell death may also be dependent upon completion of the cell's elaborate morphogenesis, and have unsuccessfully attempted by candidate gene approach to identify genes involved in tail-spike cell process extension and tail-spike cell fusion. We performed a forward genetic screen to isolate factors involved in tail-spike cell death, hoping that, based on our hypothesis, we would pick up mutants in other cell fate decisions in the tail-spike cell. We isolated several mutants, as described in Chapter 5. Several of these mutants exhibited weak defects in tail-spike cell fusion and process extension/retraction. The phenotypes of these mutants should be further characterized, and defects in tail-spike cell morphology should be carefully assessed in all isolated mutants. The screen was performed in large part to better understand cell death initiation in the tail-spike cell; however, it was designed to isolate mutants blocking tail-spike cell death, and therefore many of the mutants we isolated may be defective not in the initiation of cell death initiation but rather in its execution. Non-complementation tests with *ced-3* and *ced-4* should be performed with all mutants. In addition, the screen should be extended, as it was not performed to saturation.

We have established that *ced-3* expression is upregulated in the tail-spike cell

shortly before it dies, and propose that this upregulation provides a temporal trigger for tail-spike cell death. To better understand this mechanism, we performed a screen for regulators of *ced-3* expression in the tail-spike cell, and isolated three mutants, *ns90*, *ns114* and *ns115*. We have cloned and characterized alleles *ns114* and *ns115*, which both contain mutations in the *pal-1* gene. The gene altered by the *ns90* mutation has not yet been identified, nor has the cell death defect in *ns90* mutant animals been characterized. The *ns90* mutation is dominant; the nature of this dominance should be further defined by testing whether the *ns90*/+ phenotype is the result of haploinsufficiency, or is the result of a gain-of-function mutation in the gene mutated by *ns90*. Given the high lethality observed in mutants isolated from the screen (see Chapter 4), it may be advisable to perform further rounds of screening.

Our screen for regulators of *ced-3* expression was aimed at isolating loss-of-function mutations in activators of *ced-3* expression, or gain-of-function mutations in repressors. As an alternate approach to identifying repressors of *ced-3* expression, one could first identify the sites to which these repressors bind. We have already created twenty-one transgenic strains each of which contains a *ced-3*promoter::GFP reporter transgene with a 14 to 16 bp deletion within the conserved region of the *ced-3* promoter. We assayed tail-spike cell GFP expression in L2 worms from each of these strains, which were also homozygous for the *ced-3*(*n717*) mutation. We found that expression was blocked by three of these deletions, two of which contain binding sites for the transcriptional activator, *pal-1*. One could also take advantage of these strains to identify binding sites for repressors of *ced-3* expression. Embryos carrying the reporter transgenes could be assayed for premature *ced-3* expression in the tail-spike cell; normally, the tail-spike cell reporter only begins to be expressed in the three-fold stage embryo, several hours after the tail-spike cell is born. After putative repressor binding sites have been identified, their functional relevance may be assessed by introducing comparable deletions into a *ced-3*promoter::*ced-3* cDNA construct and

evaluating the effect of the deletions upon tail-spike cell death. If deleting these sites blocks the repression of *ced-3* transcription (ie. results in premature expression), and if *ced-3* expression is sufficient to kill the cell, we might expect premature tail-spike cell death. Transcription factors binding to these sites may be identified by a one hybrid screen, or by a candidate gene approach.

We identified the *pal-1* gene as a direct activator of *ced-3* expression. *pal-1* is a homeodomain-containing transcription factor that is expressed in several cells in the posterior region of the worm (Edgar et al., 2001). Given its broad expression, other factors must limit *pal-1* activation of *ced-3* expression to the tail-spike cell. A *pal-1* suppressor screen may identify some of these factors. Using a fluorescence-equipped dissecting microscope, in which the low levels of *ced-3* reporter expression observed in *pal-1*(*ns114*) and *pal-1*(*ns115*) mutant animals are undetectable, one could screen for animals in which *ced-3* reporter expression is restored. We expect this screen to isolate loss-of-function mutations in factors repressing *pal-1* expression or activity in the tail-spike cell, or gain-of-function mutations in *pal-1* activators; alternatively, mutations in regulators of *ced-3* expression that act independently of *pal-1* may also be isolated.

Chapter 8

Materials and Methods

Strains and general methods

All strains were grown at 20 °C on NGM agar with *E. coli* OP50 bacteria, as described by Brenner (1974). The wild-type strain was *C. elegans* variety Bristol strain N2. The following alleles were used: LGI: *ces-1*(*n703*), *ces-2*(*n732*), *ced-1*(*e1755*), *ced-12*(*k149*), *unc-73*(*e936*), *unc-40*(*e271*), *lin-17*(*n671*), *lin-44*(*n1792*); LGII: *ns90*, *unc-53*(*e404*), *eff-1*(*hy21ts*, *hy40*); LGIII: *ced-4*(*n1162*), *ced-9*(*n1950*, *n2812*), *ced-6*(*n2095*), *ced-7*(*n1892*), *pal-1*(*ns114*, *ns115*, *ok690*, *e2091*); LGIV: *ced-2*(*e1752*), *ced-3*(*n717*, *n2427*, *n2428*, *n2436*, *n2448*, *n2875*, *n2869*, *n2920*, *n2855*, *n2455*), *ced-5*(*n1812*), *unc-30*(*e191*), *ced-10*(*n1993*), *ced-2*(*e1752*), *pax-2*(*ok935*), *egl-38*(*sy294*), *unc-5*(*e53*); LGV: *egl-1*(*n1084n3082*), *vab-8*(*e1017*), *unc-76*(*e911*); LGX: *ced-13*(*tm536*, *sv32*), *ced-8*(*n1891*).

Plasmid constructions

GFP reporters were constructed by amplifying indicated regions of the *C. elegans* or *C. briggsae ced-3* promoters using the polymerase chain reaction (PCR), and cloning them into the GFP expression vector pPD95_69 (Fire et al., 1990). For construction of *C. briggsae ced-3* promoter::GFP, we amplified a 0.7kb DNA fragment from cosmid G45E19 and ligated the resulting amplicon to pPD95_69 digested with XbaI and XmaI. For *C. elegans ced-3*promoter::GFP reporter constructs, we amplified DNA

from the *C. elegans ced-3* genomic DNA construct pJ40 (Yuan et al., 1993) and ligated the resulting amplicons to pPD95_69 digested with HindIII and BamHI. For the *unc-119 C. elegans ced-3* promoter (0.35 kb)::GFP construct we subsequently used to create low-copy integrated transgenic lines, we digested *C. elegans ced-3* promoter (0.35 kb)::GFP with HindIII and ApaI, and ligated the resulting fragment to the cloning vector Bluescript pBS KS(-). This vector also contained the sequence encoding the *unc-119* gene. For construction *ced-3*promoter::*ced-3* cDNA, we amplified a 1.5 kb DNA fragment containing sequences just upstream of the *ced-3* ATG from the *ced-3* genomic DNA construct pJ40 (Yuan et al., 1993), and ligated the resulting amplicon to pPD95_69 digested with HindIII and XbaI. We then amplified a 1.5 kb DNA fragment from the *C. elegans ced-3* cDNA plasmid pS126 (Shaham and Horvitz, 1996a) and ligated the resulting amplicon to this *ced-3* promoter construct digested with XbaI and NheI. For reporter constructs containing tandem copies of sites A, B or C, we ligated oligos containing 5 tandem copies of sites A, B or C into pPD95_69 digested with SalI and BamHI. For *ced-3* inverted repeat constructs, we amplified a 0.7 kb fragment of *ced-3* cDNA (+782 to +1508) from pS126, introducing either NheI and XhoI, or KpnI and EcoRV sites at the fragment ends. A spacer DNA fragment of roughly 0.3 kb which contained part of the lac operon was amplified from the cloning vector Bluescript pBS KS(-), introducing XhoI and KpnI sites at the fragment ends. The resulting amplicons were ligated into the heat shock vectors pPD49_78 and pPD49_83. For construction of the *pal-1* genomic DNA rescue construct, we amplified a 6kb DNA fragment from the *pal-1*-containing cosmid W05E6 and ligated the resulting amplicon to the pCR 2.1-TOPO vector (Invitrogen). For construction of the *pal-1* cDNA rescue constructs, we amplified the *pal-1* cDNA from a *C. elegans* mixed stage cDNA library (S. Shaham), and ligated the resulting amplicon into pPD95_69pU digested with XhoI and SpeI. pPD95_69pU is a modified

pPD95_69 vector with SpeI, EagI and ApaI sites inserted at the 3' end of the GFP-encoding region (M. Heiman). Either the *pal-1* or the *ced-3* promoter was ligated into this *pal-1* cDNA vector. The *pal-1* promoter (-1124 to -2) was amplified from the *pal-1*-containing cosmid, W05E6, and ligated via BamHI and XhoI sites; the *ced-3* promoter (-1512 to -1) was amplified from pJ40 and ligated via BamHI and XhoI. For constructs encoding GST-fusion proteins, DNA encoding the PAL-1 full-length protein, or a C-terminal fragment including the PAL-1 homeodomain (residues 203-270) were cloned into plasmid pGEX-4T-3 (Pharmacia) using the BamHI and XhoI sites. The deletion/mutation constructs described in the text were generated using QuikChange XL Site-Directed Mutagenesis Kit (Stratagene).

Transgenic strain constructions

Transformations were performed as previously described (Mello and Fire, 1995). GFP reporter constructs were injected into *ced-3*(*n717*) animals at concentrations of 30-40 ng/μl; the plasmid pRF4, which contains the dominant marker *rol-6*(*su1006*), was used as the transformation marker (40 ng/μl). The 0.7 kb *C. briggsae ced-3* promoter::GFP extrachromosomal transgene was stably integrated by treatment with 4,5',8-trimethylpsoralen (TMP). Integrant lines stably transmitting the transgene to all progeny were isolated and further characterized. The *nsIs25* integrant, mapping to LGX, and the *nsIs23* integrant, not on LGX, were used for the experiments described here. Rescue constructs containing *ced-3* genomic DNA (pJ40) were injected into *ced-3*(*n717*); *nsIs25* animals at a concentration of 50ng/μl, using *daf-6*::RFP as the co-injection marker. The *ced-3*promoter::*ced-3* cDNA rescue construct was injected into *nsIs25*;*ced-3*(*n717*) animals at concentrations of 40 or 60 ng/μl, using *daf-6*::RFP or *daf-19*::RFP as coinjection markers (lines 1-3 and 4-6, respectively). Both *ced-3* IR heat-shock constructs (pPD49_78 and pPD49_83) were injected into *nsIs25* animals at concentrations of 50ng/μl, using the *daf-6*::RFP coinjection marker. *pal-1* rescue

constructs were injected at 10-25ng/μl using either *daf-6*::RFP or *daf-19*::NLS-RFP as co-injection markers (50ng/μl).

Generating anti-CED-3 antibodies

DNA encoding a 15-amino acid peptide corresponding to the C-terminal region of the CED-3 large subunit p17 (residues 360-374) was cloned into the pGEX-4T-3 vector (Pharmacia) using the BamHI and EcoRI sites. Rats were immunized with the resulting 15-amino acid peptide conjugated to Keyhole limpet hemocyanin (Covance), and antiserum from the termination bleed (after 3 boosts) was used in subsequent experiments.

Immunostaining using anti-caspase antibodies

Two-day old adults carrying *nsIs6*, an integrated transgene containing the *ced-13* cDNA fused to a heat-shock inducible promoter (S. Shaham, unpublished), were subjected to a one hour heat-shock, and their embryos subsequently fixed for whole-mount immunofluorescence as previously described (Finney and Ruvkun, 1990), using 1% formaldehyde. Antiserum was diluted 1:500, and tested using a FITC-labeled mouse anti-rat IgG secondary antibody. Antiserum against human/mouse caspase-3 (CM1; Srinivasan et al, 1998), *Drosophila* full-length Drice, or *Drosophila* activated Drice were incubated with embryos at a 1:250 dilution, and activity tested using FITC-labeled anti-mouse IgG secondary antibody. An antibody raised against CFI-1 (Shaham and Bargmann, 2002) was used as the positive control for the immunostaining (1:200 dilution; FITC-labeled mouse anti-rat IgG secondary antibody).

Establishing timing of reporter expression in the tail-spike cell

For individual embryos: Embryos were collected at the comma stage of embryogenesis, and allowed to develop for three hours at 20 °C. Individual embryos were subsequently

mounted in M9 buffer onto microscope slides containing 0.4 mm agar pads. Tail-spike cell morphology and *ced-3*::GFP expression were assessed at 2-4 minute intervals using a fluorescence-equipped compound microscope. Embryos were followed until visualization of the tail-spike cell corpse. For population studies: 200 embryos were synchronized at the comma stage of embryogenesis, and allowed to develop at 20 °C. Beginning 3 hours post-comma stage, groups of embryos (n=9-19) were mounted in M9 buffer onto a microscope slide and examined using a fluorescence-equipped compound microscope; 10 groups of embryos were examined at 10 equally spaced time points; embryos were assessed for expression of the *C. briggsae ced-3*::GFP reporter in tail-spike cells and corpses.

Assay for *ced-3* reporter expression

Reporter expression in the tail-spike cell was assessed in L2 animals of lines carrying the reporter constructs established as described above. At least 15 transgenic animals were examined per line.

Assay for cell death rescue in *ced-3*(*n717*) mutants

To assess rescue of the *ced-3* cell death defect, at least two transgenic lines per rescue construct were examined; 30 transgenic and five non-transgenic animals were scored per line. L3 animals were assessed for rescue of both tail-spike and pharyngeal cell death. Cell death in the pharynx was assessed as previously described (Hengartner et al., 1992).

RNAi-mediated disruption of *ced-3* expression

Adult worms carrying the *ced-3* IR constructs described above were heat-shocked for 45 minutes at 34 °C, or raised continuously at 20 °C, and their progeny separated into four groups: embryos laid 0-2 or 2-4 hours before heat shock, or 0-2 or 2-4 hours

afterwards. Inappropriate tail-spike and pharyngeal cell survival was assessed in L4 stage animals. Animals heat-shocked at the L4 stage were subjected to a 4 hour heat-shock at 34 °C.

Isolation of *ns114*, *ns115* and *ns90*

ced-3(n717); *nsIs25* animals were mutagenized with 30 mM ethyl methanesulfonate (EMS) as described by Sulston and Hodgkin. Animals were propagated in 500 ml liquid culture for 5 days. Gravid F1 adults were harvested, bleached, and their progeny incubated overnight in M9 buffer. F2s were plated onto 9 cm plates, and screened as early larvae for the absence of tail-spike cell GFP expression. 32,000 F2s were screened, and 621 candidate mutants were isolated. Mutant alleles *ns90*, *ns114*, and *ns115* bred true, and were further characterized.

Expression of GST fusion proteins

Expression of the GST-PAL-1 FL and GST-PAL-1 HD proteins was induced in *E. coli* BL21. Fusion proteins were isolated by passing supernatant of bacterial sonicate over a Glutathione Sepharose 4B column (Amersham) and elution with soluble glutathione.

Electrophoretic Mobility Shift Assay

30 ng of fusion protein were incubated for 15 minutes at room temperature with 0.25 ng of double stranded 32P-labelled oligonucleotides (B WT: 5'CATCATAAACTTTTTTTTCCGC) and unlabelled competitor oligonucleotides (B MUT: CATACGCCCCTTTTTTTTCCGC, C WT: GCAATAAACCGGCCAAAAACTT, C MUT: GCACGCCCCCGGCCAAAAACTT, A WT: CTACAATCCACTTTCTTTTCTC or A MUT: CTATTTTCTTTCACCTAACCTC) in 20μl of a solution containing 2 mM $MgCl_2$, 50 μg/mL bovine serum albumin, 10 mM Tris (pH 7.5), 1 mM EDTA, 40

mM KCl, 1 mM DTT and 5% glycerol. Oligonucleotides were end-labeled with ^{32}Pγ-dATP by incubation with T4 polynucleotide kinase. Unbound ^{32}Pγ-dATP nucleotide was removed using the Stratagene NucTrap Probe Purification Column. Binding reactions were run on a 4% polyacrylamide gel in 0.5X TBE buffer.

Isolation of *ns16*, *ns17*, *ns18*, *ns19*, *ns38*, *ns39*, *ns40*, and *ns41*

nsIs23 or *nsIs25* animals were mutagenized with 30 mM ethyl methanesulfonate (EMS) as described by Sulston and Hodgkin (1988). F1 progeny were transferred to 5 cm NGM agar plates; 2 animals were placed on each plate. F2s were screened as early larvae for inappropriate tail spike survival as determined by GFP reporter expression. 1,500 F2s were screened, and 89 candidate mutants were isolated. Mutant alleles *ns16*, *ns17*, *ns18*, *ns19*, *ns38*, *ns39*, *ns40*, and *ns41* bred true and were further characterized.

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