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Timing of the onset of a developmental cell death is controlled by transcriptional induction of the *C. elegans ced-3* caspase-encoding gene

Carine W. Maurer, Michael Chiorazzi and Shai Shaham*

Temporal control of programmed cell death is necessary to ensure that cells die at only the right time during animal development. How such temporal regulation is achieved remains poorly understood. In some *Caenorhabditis elegans* somatic cells, transcription of the *egl-1*/BH3-only gene promotes cell-specific death. The EGL-1 protein inhibits the CED-9/Bcl-2 protein, resulting in the release of the caspase activator CED-4/Apaf-1. Subsequent activation of the CED-3 caspase by CED-4 leads to cell death. Despite the important role of *egl-1* transcription in promoting CED-3 activity in cells destined to die, it remains unclear whether the temporal control of cell death is mediated by *egl-1* expression. Here, we show that *egl-1* and *ced-9* play only minor roles in the death of the *C. elegans* tail-spike cell, demonstrating that temporal control of tail-spike cell death can be achieved in the absence of *egl-1*. We go on to show that the timing of the onset of tail-spike cell death is controlled by transcriptional induction of the *ced-3* caspase. We characterized the developmental expression pattern of *ced-3*, and show that, in the tail-spike cell, *ced-3* expression is induced shortly before the cell dies, and this induction is sufficient to promote the demise of the cell. Both *ced-3* expression and cell death are dependent on the transcription factor PAL-1, the *C. elegans* homolog of the mammalian tumor suppressor gene Cdx2. PAL-1 can bind to the *ced-3* promoter sites that are crucial for tail-spike cell death, suggesting that it promotes cell death by directly activating *ced-3* transcription. Our results highlight a role that has not been described previously for the transcriptional regulation of caspases in controlling the timing of cell death onset during animal development.

KEY WORDS: Cell death onset, Caspase, *ced-3*, *C. elegans*, Tail-spike cell

INTRODUCTION

Programmed cell death (PCD) plays an important role in organismal development; it enables multicellular organisms to regulate tissue and organismal size by serving to counter proliferative forces, and destroys structures when they become irrelevant, as occurs during amphibian and insect metamorphosis (reviewed in Jacobson et al., 1997). Defects in programmed cell death underlie a variety of pathological processes, including autoimmune and neurodegenerative disorders and cancer (reviewed in Hanahan and Weinberg, 2000; Yuan and Yankner, 2000). The nematode *C. elegans* has proven to be instrumental in deciphering the molecular basis of PCD. A genetic pathway governing the execution of cell death was first identified in *C. elegans* (Metzstein et al., 1998), and the machinery responsible for executing cell death in this animal is conserved in many metazoans. In most *C. elegans* cells, mitochondrially-associated CED-9 protein (similar to vertebrate Bcl-2) binds to and sequesters CED-4 (similar to vertebrate Apaf-1), thus inhibiting cell death by blocking CED-4-mediated activation of the CED-3 caspase (Chen et al., 2000). Release of CED-4 from the mitochondria allows this protein to promote proteolytic activation of CED-3, which leads to cellular demise (Shaham and Horvitz, 1996a; Chen et al., 2000; Yan et al., 2004).

Although a conserved cell death execution machinery has been well characterized, the molecular events that determine the timing of cell death onset remain poorly understood. In a subset of somatic cells in *C. elegans*, *egl-1* is believed to be a crucial mediator of the life versus death decision. *egl-1* acts upstream of *ced-9* to promote cell death (Conradt and Horvitz, 1998) by antagonizing the interaction between CED-9 and CED-4, allowing the release of CED-4 from mitochondria (Chen et al., 2000). The 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 onset. 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 death (Conradt and Horvitz, 1999; Ellis and Horvitz, 1991; Metzstein et al., 1996; Metzstein and Horvitz, 1999; Thellmann et al., 2003; Hoeppner et al., 2004; Liu et al., 2006).

Although control of the timing of *egl-1* expression is thought to be the main avenue regulating the onset of cell death in *C. elegans*, transcription of the gene has not been examined at high temporal resolution, leaving open the possibility that, although *egl-1* expression is necessary for cell death to occur, it may not be the trigger for the process. Two observations are consistent with the notion that induction of *egl-1* transcription may not be the only temporal cue regulating the onset of cell death. First, physiological cell death in the *C. elegans* germ line occurs independently of *egl-1* (Gumienny et al., 1999) or of the related BH3-only protein CED-13 (Schumacher et al., 2005), suggesting that the onset of cell death must be regulated by other factors in this tissue. Second, 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 some cells destined to die, but not in cells destined to live (Hengartner and Horvitz, 1994). Because *egl-1* requires *ced-9* to promote cell death, *egl-1* cannot be the determinant of cell death onset in these cells, which lack *ced-9* activity. Thus, other mechanisms controlling the timing of cell death onset must exist; their nature, however, is unknown.

Laboratory of Developmental Genetics, The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA.

*Author for correspondence (e-mail: shaham@rockefeller.edu)

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Caspases play important roles in programmed cell death as key mediators of cellular destruction. These proteases are synthesized as pro-proteins with weak proteolytic activity, and intricate post-translational regulation ensures the tight control of their activities (reviewed by Abraham and Shaham, 2004; Shi, 2004). Caspases are expressed in both living and dying cells in *C. elegans* (Shaham and Horvitz, 1996a; Shaham et al., 1999), and this expression is required for the execution of PCD. 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 (also known as Nc – FlyBase) and of the effector caspase Drice (also known as Ice – FlyBase) 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 in 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. In these systems, caspase expression, although crucial 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.

Here, we report that the death of the *C. elegans* tail-spike cell depends only partially on the *egl-1* and *ced-9* genes, suggesting that other means of exerting temporal control on the death of this cell must exist. We demonstrate that the induction of *ced-3* caspase transcription, shortly before morphological features of death are evident, plays a crucial role in controlling the onset of PCD in the tail-spike cell, 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*, which is required for both the expression of *ced-3* in the tail-spike cell and for tail-spike cell death, and in vitro studies demonstrate that PAL-1 is able to bind the *ced-3* promoter at sites that are crucial for both processes, suggesting that *pal-1* directly promotes *ced-3* expression. The PAL-1 protein is similar to the mammalian protein Cdx2, which promotes intestinal development and, when mutated, can result in intestinal tumors (Chawengsaksophak et al., 1997; Aoki et al., 2003; Bonhomme et al., 2003). Our results unveil a novel mechanism for controlling the timing of programmed cell death by the transcriptional regulation of caspases.

MATERIALS AND METHODS

Strains and general methods

All strains were grown at 20°C on NGM agar with *E. coli* OP50 bacteria, as previously described (Brenner, 1974). The wild-type strain was *C. elegans* variety Bristol strain N2. The following alleles were used:

LGI: *smg-1(r861)*;

LGIII: *ced-4(n1162)*, *ced-9(n1950, n2812)*, *pal-1(ns114, ns115, ok690)*, *bcIs1*;

LGIV: *ced-3(n717, n2427, n2436)*, *ced-5(n1812)*, *unc-30(e191)*;

LGV: *egl-1(n1084n3082)*, *bcIs37*;

LGX: *ced-13(tm536, sv32)*, *nsIs25*.

Plasmid constructions

Green fluorescent protein (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 the *C. briggsae ced-3* promoter::GFP, we amplified a 0.7 kb DNA fragment from cosmid G45E19 and ligated the resulting amplicon to pPD95.69 digested with *Xba*I and *Xma*I. To generate the *C. elegans ced-3* promoter (0.35kb)::GFP fusion, we amplified DNA from the *C. elegans ced-3* genomic DNA construct pJ40 (Yuan et al., 1993) and ligated the resulting amplicon to pPD95.69 digested with *Hin*dIII and *Bam*HI. For creation of *C. elegans ced-3* promoter (1.5 kb)::GFP, we amplified DNA from pJ40 and ligated the resulting amplicon to pPD95.69 digested with *Hin*dIII and *Bam*HI. The *ced-3* expression reporter used in Fig. 2 was made as follows: GFP was amplified by PCR from plasmid pPD95.69 using primers containing *Mlu*I sites followed by the GFP start or stop codons, and was inserted into the *Mlu*I site of plasmid pJ40. This construct encodes a fusion protein containing 69 amino acids of CED-3 protein fused to GFP. For construction of the *pal-1* genomic-DNA rescue construct, we amplified a 6 kb DNA fragment from cosmid W05E6 and ligated the resulting amplicon to the pCR 2.1-TOPO vector (Invitrogen). For construction of *ced-3* promoter::*ced-3* cDNA, we amplified a 1.5 kb DNA fragment containing sequences immediately upstream of the *ced-3* ATG from pJ40, and ligated the resulting amplicon to pPD95.69 digested with *Hin*dIII and *Xba*I. 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 *Xba*I and *Nhe*I. The deletion constructs described in Fig. 3 and in Fig. S1B in the supplementary material, and the mutant B and C 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 (containing 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 extra-chromosomal transgene was stably integrated by treatment with 4,5′,8-trimethylpsoralen (TMP). Integrant lines stably transmitting the transgene to all progeny were isolated and characterized further. 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 50 ng/μL, using *daf-6*::RFP as the co-injection marker. *pal-1* rescue constructs were injected at 25 ng/μL using either *daf-6*::RFP or *daf-19*::NLS-RFP as co-injection markers.

Establishing timing of reporter expression in the tail-spike cell

Individual embryos were collected at the comma stage of embryogenesis and were allowed to develop for 3 hours at 20°C. Embryos were subsequently mounted in M9 buffer onto microscope slides containing 0.4 mm agar pads. Tail-spike cell *ced-3*::GFP expression and cell morphology were assessed at 2-4 minute intervals using a fluorescence-equipped compound microscope. Embryos were followed until visualization of the tail-spike cell corpse.

Assay for *ced-3* reporter expression

Reporter expression in the tail-spike cell was assessed in L2 animals of lines carrying deletion 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).

Isolation of *ns114* and *ns115*

ced-3(*n717*); *nsIs25* animals were mutagenized with 30 mM ethyl methanesulfonate (EMS) as described previously (Sulston and Hodgkin, 1988). 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 were screened as early larvae for the absence of tail-spike cell GFP expression. In total, 32,000 F2s were screened and 621 candidate mutants were isolated. Mutant alleles *ns90*, *ns114*, and *ns115* bred true, and were characterized further.

Electrophoretic mobility shift assay

DNA encoding a C-terminal fragment including the PAL-1 homeodomain (residues 203-270) was cloned into plasmid pGEX-4T-3 (Pharmacia) using the *Bam*HI and *Xho*I sites. The resulting plasmid was used to express the

GST-PAL-1HD protein in *E. coli* BL21. Fusion protein was isolated by passing supernatant of bacterial sonicate over a Glutathione Sepharose 4B column (Amersham) and by elution with soluble glutathione. A total of 30 ng of fusion protein was incubated for 15 minutes at room temperature with 0.25 ng of double-stranded ^{32}P-labeled oligonucleotides (B WT: 5′-CATCATAAACTTTTTTTTCCGC-3′; C WT: 5′-GCAATAAACCGGCCAAAAACTT-3′; B MUT: 5′-CATCATCCACTTTTTTTTCCGC-3′; C MUT: 5′-GCAATCCACCGGCCAAAAACTT-3′) and unlabeled competitor oligonucleotides in 20 μl of a solution containing 50 μg/mL bovine serum albumin, 10 mM Tris (pH 7.5), 1 mM EDTA, 100 mM KCl, 0.1 mM DTT, 5% glycerol and 100 ng of salmon sperm DNA. 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 0.5×TBE buffer. Each mobility shift assay described in the paper was repeated at least three times. For Fig. 6C, the mutant C oligo used was 5′-GCACGCCCCCGGCCAAAAACTT-3′.

RESULTS

Identification of a tail-spike cell reporter

To understand better how cell death is temporally regulated, we investigated the mechanisms underlying the death of the *C. elegans* tail-spike cell. The tail-spike cell dies more than 5 hours after it is born, surviving ten times longer than most cells destined to die in *C. elegans* (Sulston and Horvitz, 1977; Sulston et al., 1983), thus facilitating analysis of the kinetics of the onset of cell death. 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) (Fig. 1A). Thus, precise control of the timing of tail-spike cell death onset may be important for *C. elegans* tail development. In *Drosophila* and vertebrates, many cells that die during development exhibit obvious differentiated features and can live long after they are born (Meier et al., 2000). Thus, tail-spike cell death may be more similar to developmental cell deaths that occur during development in *Drosophila* and vertebrates than to other cell deaths that occur in *C. elegans*.

To study tail-spike cell death, we developed reagents to label the cell. We noticed that a 1.5 kb region of 5′ *ced-3* promoter sequences, when fused to a *ced-3* cDNA, could provide *ced-3* function to the tail-spike cell (see below), suggesting that this promoter fragment could be used to drive reporter gene expression in this cell. Indeed, the same promoter region was sufficient to drive expression of *lacZ* (S.S., PhD thesis, Massachusetts Institute of Technology, 1995) (Shaham et al., 1999) or GFP reporter transgenes in the tail-spike cell (Fig. 1C,D). However, these transgenes also occasionally blocked tail-spike cell death (see Table S1 in the supplementary material), limiting their use. A similar transgene consisting of a 0.7 kb *ced-3* promoter fragment from the related nematode *C. briggsae* fused to the GFP-encoding gene proved to be a better reporter because it also marked the tail-spike cell but did not promote inappropriate cell survival (Table 1A and see Table S1 in the supplementary material).

egl-1 and *ced-9* play minor roles in tail-spike cell death

Using genomically integrated *C. briggsae ced-3* promoter::GFP reporter transgenes (*nsIs25* or *nsIs23*), we examined whether tail-spike cell death was dependent upon the known cell death execution machinery. As expected, we found that cell death was completely blocked in animals homozygous for the *ced-3*(*n717*) or *ced-4*(*n1162*)-null alleles (Table 1A). 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 cells of the anterior pharynx (Table 1). To confirm this partial effect on tail-spike cell death, we examined *egl-1*(*n1084n3082*) mutants that were 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). These corpses do not form if cell death is prevented (Ellis and Horvitz, 1986). Consistent with our GFP results, a majority (24 out of 43, 56%) of *ced-5*(*n1812*); *egl-1*(*n1084n3082*) first larval-stage (L1) animals still exhibited a persistent tail-spike cell corpse (see below), supporting the notion that *egl-1* has only a partial role in promoting tail-spike cell death. Tail-spike cell death was unaffected by null mutations in the *ced-13* gene (Table 1A), the only other characterized *C. elegans* BH3-domain-only gene (Schumacher et al., 2005), and *ced-13* mutations did not enhance the defects observed in *egl-1* mutants (Table 1A), indicating that the partial block of tail-spike cell death in *egl-1* mutants was not due to redundancy with *ced-13*.

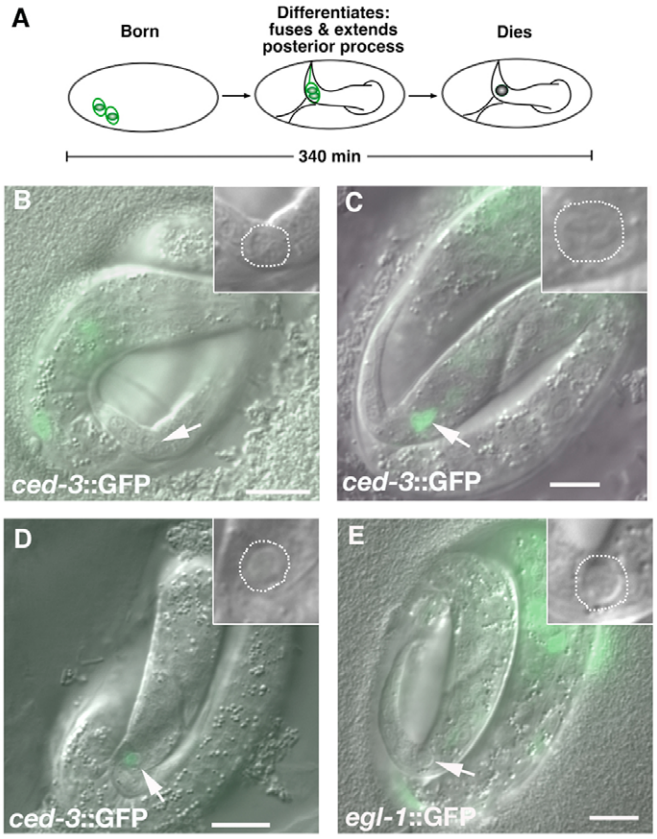

Fig. 1. Expression of *ced-3* promoter::GFP transgenes is induced shortly before tail-spike cell death. (**A**) Cartoon showing the tail-spike cell at different stages of development. Filled green ovals, tail-spike cell nuclei; gray oval, dying tail-spike cell corpse. Ball-stage embryo (left); threefold-stage embryo (center and right). (**B-D**) Merged DIC and GFP images of threefold-stage embryos expressing a 1.5 kb *C. elegans ced-3* promoter::GFP reporter transgene (*nsEx723*). (B) Embryo before the onset of tail-spike cell GFP expression (arrow and inset: binucleate tail-spike cell). (C) Embryo at onset of tail-spike cell GFP expression (arrow and inset: bi-nucleate tail-spike cell). (D) Embryo approximately 31 minutes after the onset of tail-spike cell GFP expression (arrow and inset: dying tail-spike cell corpse). (**E**) Merged DIC and GFP image of a threefold-stage embryo expressing an *egl-1* promoter::GFP integrated transgene (*bcIs1*). Arrow and inset: tail-spike cell corpse lacking GFP. Scale bars: 10 μm.

Table 1. Effects of mutations in the cell death machinery on tail-spike and pharyngeal cell death

Genotype[a]	% surviving tail-spike cells[b]	Average no. extra cells in anterior pharynx
A. *egl-1* is partially required for tail-spike cell death		
Wild type	0±0[c]	0.5±0.2[d]
ced-3(n717)	100±0	10.6±1.5
ced-4(n1162)	100±0	11.1±1.1
egl-1(n1084n3082)	30±6	11.1±1.5
ced-13(tm536)	0±0	0.1±0.4
ced-13(sv32)	0±0	0.2±0.4
egl-1(n1084n3082); ced-13(tm536)	41±4	11.6±1.4
B. *egl-1* inhibits *ced-9* to promote tail-spike cell death		
ced-3(n2427)	0±0	2.1±1.2
ced-9(n2812); ced-3(n2427)	0±0	6.5±1.1
ced-3(n2427); egl-1(n1084n3082)	100±0	11.2±1.8
ced-9(n2812); ced-3(n2427); egl-1(n1084n3082)	0±0	5.2±1.8
C. *ced-9* lacks death-promoting activity in the tail-spike cell		
ced-3(n2436)	59±8	6.3±1.7
ced-9(n2812); ced-3(n2436)	63±9	10.5±1.8
D. A *ced-9(gf)* mutation weakly blocks tail-spike cell death		
ced-9(n1950gf)	3±3	10.3±1.3
ced-9(n1950gf); ced-3(n2427)	50±9	11.5±1.1
ced-9(n1950gf); ced-3(n2436)	93±5	11.0±1.8
ced-5(n1812)	3±3	0.2±0.5
ced-9(n1950gf); ced-5(n1812)	27±8	10.3±1.6
ced-5(n1812); ced-3(n2427)[e]	17±7	5.1±1.8
ced-9(n1950gf); ced-5(n1812) ced-3(n2427)[e]	73±8	11.1±1.5
ced-9(n1950gf); egl-1(n1084n3082)	33±8	10.3±1.7

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

To determine whether *egl-1* is expressed in the tail-spike cell, we examined the expression of genomically integrated *egl-1* promoter::GFP transgenes (*bcIs1* and *bcIs37*) that have been previously used to show *egl-1* expression in dying cells (Conradt and Horvitz, 1999; Thellman et al., 2003; Liu et al., 2006). We failed to detect GFP expression in 9/9 (*bcIs1*) and 6/6 (*bcIs37*) tail-spike cell corpses (Fig. 1E), and in 27/27 (*bcIs1*) and 21/21 (*bcIs37*) tail-spike cells observed 30-60 minutes before corpse formation. An identical transgene, in which GFP sequences were replaced with *egl-1* coding sequences, fully rescued the tail-spike cell survival defect in *ced-3*(*n2427*); *egl-1*(*n1084n3082*) mutants (147/152 transgenic animals were rescued in three lines that were observed; Table 1). These results suggest that *egl-1* expression in the tail-spike cell is low, consistent with the partial genetic role of *egl-1* in tail-spike cell death.

To determine whether the partial pro-apoptotic activity of *egl-1* was mediated by the *ced-9* gene, we tested whether a *ced-9* loss-of-function (lf)-null mutation could suppress the *egl-1*(*n1084n3082*) tail-spike cell death defect. Animals homozygous for *ced-9*(lf) alleles do not survive, and this lethality is rescued by mutations in *ced-3* (Hengartner et al., 1992). Whereas *ced-3*(weak lf); *egl-1*(lf) double mutants exhibited a fully penetrant tail-spike cell death defect, *ced-9*(lf); *ced-3*(weak lf); *egl-1*(lf) triple mutants showed no defect in tail-spike cell death (Table 1B). This result demonstrates that, as in other cells destined to die, EGL-1 exerts its pro-apoptotic function in the tail-spike cell by inhibiting the anti-apoptotic functions of CED-9.

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 as well as death-preventing activities. Surprisingly, we found that tail-spike cell death proceeded normally in *ced-9*(*n2812* lf); *ced-3*(*n2427* weak lf) double mutants (Table 1B), and that the tail-spike cell death defect in *ced-9*(*n2812*); *ced-3*(*n2436* weak lf) double mutants was identical to that of *ced-3*(*n2436* weak lf) single mutants (Table 1C). These results indicate that *ced-9* lacks death-promoting activity in the tail-spike cell. We also found that, unlike other somatic cells, tail-spike cell death was only weakly affected by the *ced-9*(*n1950*) gain-of-function (gf) 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 1D), and it only partially enhanced the cell death defects observed in *ced-3*(weak lf) and *ced-5*(lf) backgrounds (Table 1D). Previous studies argue 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 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 (see Discussion).

Induction of *ced-3* transcription determines the time of tail-spike cell death onset

Previous studies in *C. elegans* suggested that the regulation of cell death initiation may be achieved by direct or indirect post-translational regulation of caspases by, for example, transcriptional regulation of the *egl-1* gene (Conradt and Horvitz, 1999; Metzstein and Horvitz, 1999; Thellmann et al., 2003; Liu et al., 2006). Similar mechanisms have been proposed in vertebrates and in *Drosophila* (Meier et al., 2000). However, the partial effects of *egl-1* mutations

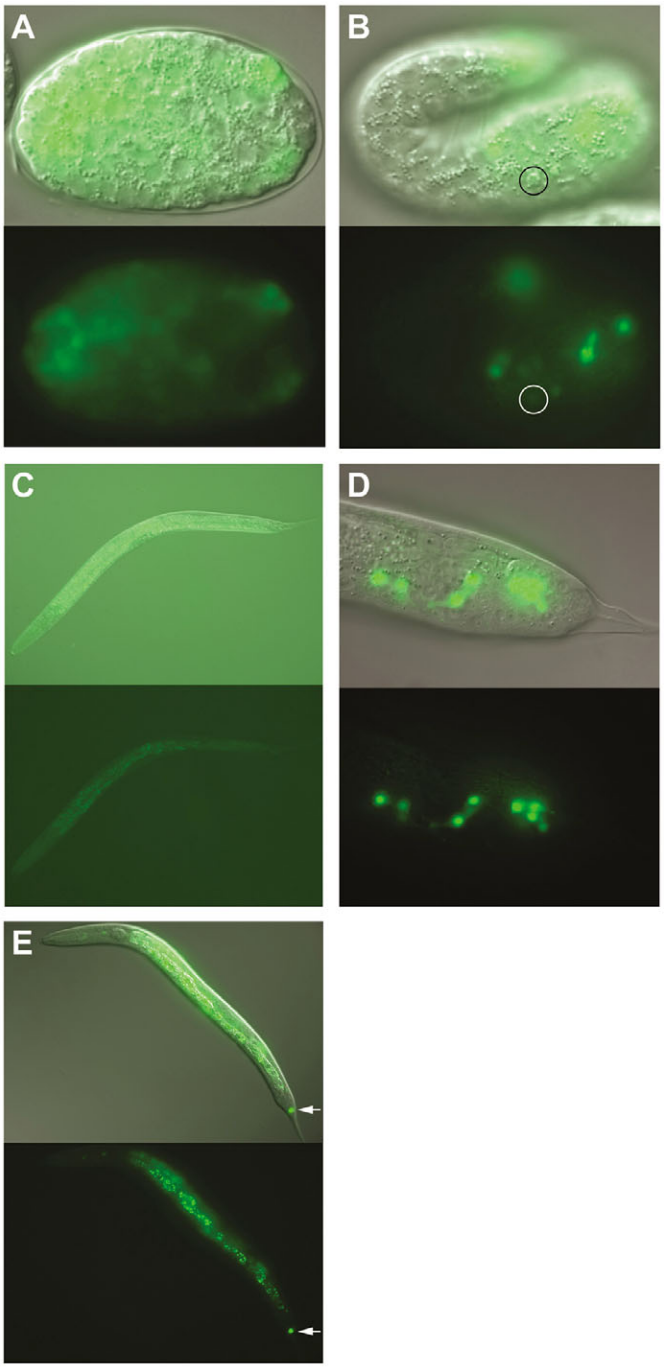

Fig. 2. *ced-3* is transcribed in many cells during developmental periods of cell death. Top and bottom panels for each image are DIC+fluorescence and fluorescence-alone images, respectively. (**A**) Image of a *smg-1*(*r861*) embryo expressing a transgene consisting of the full rescuing (7.6 kb) *ced-3* genomic locus, into which GFP with a nuclear localization signal near the start codon was inserted (see Materials and methods). (**B**) Same as A. Notice cell corpse (circled) weakly expressing GFP. (**C**) A *smg-1*(*r861*) larva containing the same transgene as in A. Notice the absence of GFP expression. The observed fluorescence is caused by autofluorescence from gut granules. (**D**) A *smg-1*(*r861*) L4 male larva containing the same transgene as in A. Notice the bright expression in several cells in the tail. None of these cells is fated to die. (**E**) A *smg-1*(*r861*); *ced-3*(*n717*) larva containing the same transgene as in A. Notice the GFP expression in the tail-spike cell (arrows).

on tail-spike cell death and the unusual effects of *ced-9* mutations in this cell strongly suggested to us that a mechanism independent of *egl-1* must control the timing of the onset of tail-spike cell death. Such a mechanism must function upstream of or in parallel to the *ced-3* caspase, because a *ced-3*(strong lf) allele completely blocked tail-spike cell death (Table 1A). Unexpectedly, several lines of evidence implicated transcription of *ced-3* as a potential step in regulating the timing of tail-spike cell death (see below).

To characterize the general pattern of *C. elegans ced-3* transcription, we constructed a plasmid containing 7.6 kb of *ced-3* genomic rescuing DNA into which we inserted sequences encoding GFP at a position 207 nucleotides downstream of the *ced-3* start codon (see Materials and methods). This construct contains all the *ced-3* regulatory sequences sufficient to rescue *ced-3* mutants. Because a stop codon was introduced immediately after the GFP sequence, the GFP message expressed from this plasmid is predicted to contain a long 3′ UTR consisting of most of the *ced-3* coding and non-coding sequences. To ensure that the construct was properly expressed, we introduced it as an extra-chromosomal transgene into *smg-1*(*r861*) animals, which have previously been shown to allow the expression of transcripts with aberrant 3′ UTRs (Wilkinson et al., 1994). Neither the transgene nor the *smg-1*(*r861*) allele had any effect on programmed cell death in the pharynx (data not shown) and, as expected, the transgene occasionally perturbed tail-spike cell death (see above; data not shown). Three transgenic lines were scored.

Examination of transgenic embryos at different developmental stages revealed that GFP was expressed in all or nearly all cells, except intestinal cells and their precursors, starting at around 100-150 minutes post-fertilization and continuing throughout the comma stage of embryogenesis (Fig. 2A). Reporter expression disappeared after the threefold embryonic stage, and only 2-3 cells expressed GFP faintly in larva and adults (Fig. 2C). These cells, one of which was probably the head mesodermal cell, were present in the head and did not reliably express GFP. Reporter expression was similar between males and hermaphrodites, except that, in males, intense staining was observed in the tails of L4 animals (Fig. 2D). Interestingly, a bout of cell death occurs in the late L4 in the male during tail morphogenesis (Sulston and Horvitz, 1977).

Most cells expressing GFP in embryos or in the male tail were not cells destined to die, consistent with previous functional studies (Shaham and Horvitz, 1996a); and, although we could detect GFP staining in dying cells, staining was generally weaker than in other cells (Fig. 2B). GFP expression in dying cells could reflect de novo transcription or residual GFP expressed in a precursor cell and perduring in the dying cell. To distinguish between these possibilities, we observed the GFP expression pattern in *smg-1*(*r861*); *ced-3*(*n717*) animals carrying the same *ced-3*::GFP transgene described above. GFP expression was nearly identical to that observed in *smg-1*(*r861*) animals. Specifically, expression was not detected in inappropriately surviving cells in larvae or adults (Fig. 2E) (although see below for tail-spike cell), as would be predicted if *ced-3* transcription is normally induced in these cells. These results are consistent with the hypothesis that *ced-3* is not actively transcribed in cells destined to die, but is transcribed in their precursors. Supporting this notion, of the 131 somatic cells that die during hermaphrodite development, 18 cells die in the L1 and L2 stages. No expression of the *ced-3*::GFP transgene was ever detected in these cells in either *smg-1*(*r861*) or *smg-1*(*r861*); *ced-3*(*n717*) animals, even though the *ced-3* genomic region we used to construct the GFP reporter was sufficient to rescue the inappropriate survival of these cells in *ced-3*(*n717*) mutants (S.S., unpublished). The 18 cells that die postembryonically are descendents of the W, Pn, T and

ABpl/rapapaa blast cells that are born at the 200-cell stage, prior to the comma stage. ABpl/rapapaa are the immediate precursors of the QL/R and V5L/R cells. As mentioned above, these blast cells do express GFP in the embryo after they are born. Thus, it is possible that *ced-3* is normally expressed in the blast cells of postembryonic cells fated to die, and that perduring *ced-3* RNA or protein eventually leads to their death.

Unlike other cells fated to die, however, GFP expression did persist in the tail-spike cell throughout development and adulthood in *smg-1*(*r861*); *ced-3*(*n717*) mutants (Fig. 2E), suggesting that *ced-3* is normally actively transcribed in the tail-spike cell. Consistent with this observation, as described above, a 1.5 kb *C. elegans ced-3* promoter::GFP reporter expressed in many *C. elegans* cells occasionally blocked tail-spike cell death (presumably by titering a limiting transcription factor); although it had no effect on other cell deaths we scored (see Table S1 in the supplementary material). These results suggest that *ced-3* transcription in the tail-spike cell could be important in regulating the timing of tail-spike cell death onset.

To test this hypothesis, we sought to follow the kinetics of *ced-3* expression in the tail-spike cell in greater detail. Because a good antibody recognizing CED-3 has been difficult to generate (C.W.M. and S.S., unpublished), and because RNA in situ hybridization cannot be used reliably to detect *ced-3* expression (S.S., unpublished), we could not directly follow the production of *ced-3* mRNA or protein in the tail-spike cell. We decided, therefore, 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), as were described in the first subsection of the Results. Intriguingly, whereas expression of either reporter was detected in many cells, and throughout embryogenesis, in a pattern similar to that shown in Fig. 2 (data not shown), expression in the tail-spike cell was only observed during the threefold stage of embryogenesis (Fig. 1B,C), 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±s.d; *n*=6; *C. elegans* reporter) or 24.8±2.5 minutes (*n*=5; *C. briggsae* reporter) prior to visible signs of cell death (Fig. 1B-D). Similar findings were obtained by scoring *ced-3* expression at specific time points in populations of synchronized embryos (*n*=10-15 embryos per time point, with ten equally spaced time points examined between 3-5 hours after the embryonic comma stage; data not shown). The finding 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. We found that this construct was sufficient to promote tail-spike cell death. For example, in three different lines, tail-spike cell death was rescued in 29/30, 26/30 and 28/30 animals examined, indicating that *ced-3* expression at this late stage in the development of the tail-spike cell is sufficient to promote the demise of the cell, and supporting the hypothesis that *ced-3* transcription may normally 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.

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* (Fig. 3A and see Fig. S1A in the supplementary material), and is sufficient to drive GFP reporter expression in the tail-spike cell (Fig. 3B). To identify 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 (Fig. 3B and see Fig. S1B in the supplementary material). As shown in Fig. 3B, three regions (A, B and C) 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* (Fig. 3A). Although deleting regions A, B or C individually in the larger 1.5 kb *ced-3* promoter::GFP transgene did not fully block GFP expression (although a significant partial effect was seen upon deletion of region B), double deletions abolished GFP expression in the tail-spike cell

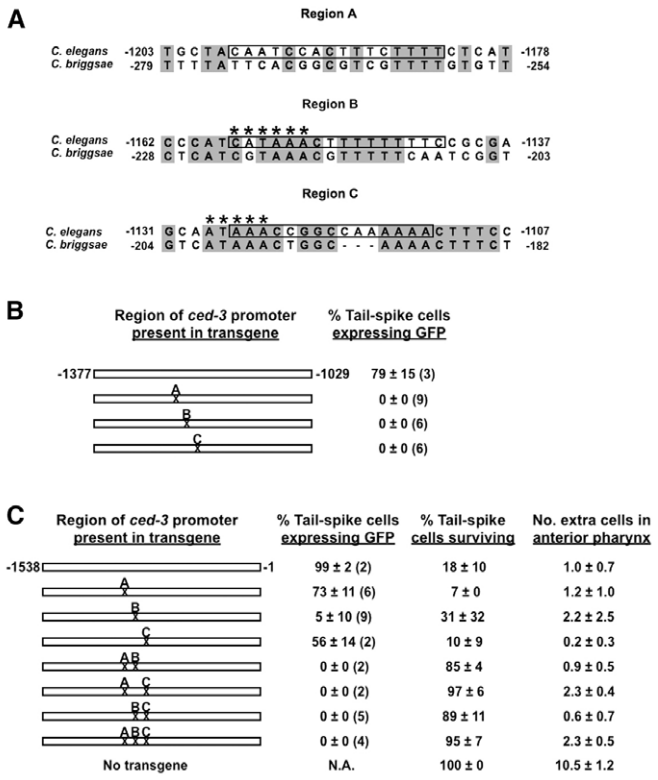

Fig. 3. Three *ced-3* promoter elements act redundantly and specifically to regulate cell death and *ced-3* expression in the tail-spike cell. (**A**) Alignment of the A, B and C promoter regions from the nematodes *C. elegans* and *C. briggsae*. Conserved nucleotides are shaded; boxed nucleotides are deleted in **B** and **C**; consensus nucleotides of caudal/Cdx2-binding sites are indicated by asterisks above the relevant nucleotides; numbers indicate the positions relative to the *ced-3* start codon. (B,C) Regions A, B and C are required for GFP reporter expression and for the rescue of the *ced-3*(*n717*) cell death defect in the tail-spike cell. All experiments were performed in *ced-3*(*n717*) mutants. Regions A, B or C were deleted in the context of the conserved 349 bp *C. elegans ced-3* promoter (B), the 1.5 kb *C. elegans ced-3* promoter (C, left data column), or of pJ40, a plasmid containing *C. elegans ced-3* genomic DNA (C, middle and right data columns). Reporter expression and cell death rescue were assessed as described in the Materials and methods section. x, deleted region; % Tail-spike cells expressing GFP, average±s.e.m. (number of transgenic lines examined); % Tail-spike cells surviving, average±s.e.m. (2-3 transgenic lines); No. extra cells in anterior pharynx, average±s.d. (2-3 transgenic lines); N.A., not applicable.

(Fig. 3C), suggesting that these sequences function redundantly to control *ced-3* expression in this 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 did rescue inappropriate cell survival in the anterior pharynx (Fig. 3C), 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 controlling the timing of tail-spike cell death initiation.

Mutations reducing *ced-3* transcription in the tail-spike cell block its death

To identify regulators of *ced-3* expression in the tail-spike cell, F2 progeny of mutagenized *ced-3*(*n717*); *nsIs25* animals were screened for the loss of tail-spike cell GFP expression. From 16,000 haploid genomes examined, we recovered three independent mutants (*ns90*, *ns114* and *ns115*) defective in tail-spike cell GFP expression. All three mutants exhibited a wild-type reporter expression pattern in other cells examined (data not shown). *ns114* and *ns115* are discussed here, and *ns90* will be described elsewhere.

ns114; *ced-3*(*n717*) animals exhibited weak (42%, *n*=88, three 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* (Fig. 4A,B), leading us to hypothesize 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 out 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 (data not shown). This proportion is probably an underestimate of cell survival, because about 50% of cells did not express GFP at all and were not scored. Second, whereas 31 out of 33 (94%) of *ced-5*(*n1812*) animals exhibited a persistent tail-spike cell corpse, a persistent cell corpse was only seen in 20 out of 46 (43%) *ns114*; *ced-5*(*n1812*) animals (Fig. 4C,D), further indicating that this mutation blocked tail-spike cell death. Interestingly, the tail-spike cell death defect was greatly enhanced in *ns114*; *egl-1*(*n1084n3082*) double mutants compared with either single mutant alone (Fig. 4D), suggesting that *ns114* affected a gene acting in parallel to *egl-1* to promote tail-spike cell death. Consistent with this result, 10 out of 32 (31%) *ns114*; *ced-9*(*n2812* lf); *ced-3*(*n2427*) animals had inappropriately surviving tail-spike cells, as scored in cells weakly expressing GFP, whereas *ced-9*(*n2812*); *ced-3*(*n2427*) mutants had no tail-spike cell survival (Table 1), indicating that *ns114* must affect a gene that functions in parallel to *ced-9*.

In addition to defects in *ced-3* expression and tail-spike cell death, some *ns114* animals exhibited defects in tail-spike cell fusion (see Fig. S2A in the supplementary material) and some possessed a mild tail deformity (see Fig. S2B in the supplementary material). Tail-spike cell death defects were consistently observed 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.

PAL-1 controls the 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

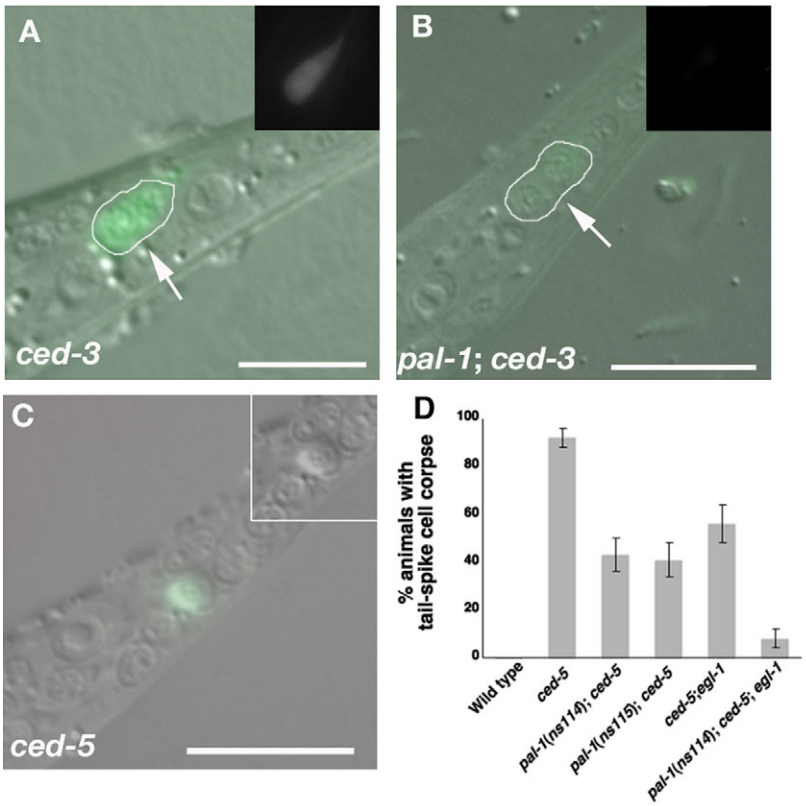

Fig. 4. *pal-1* regulates *ced-3* expression and cell death in the tail-spike cell. (**A**) A 0.7 kb *C. briggsae ced-3* promoter::GFP reporter (*nsIs25*) is expressed in a surviving tail-spike cell (arrow; inset: GFP only) of a *ced-3*(*n717*) L2 larva. (**B**) Same reporter as in A in a *pal-1*(*ns114*); *ced-3*(*n717*) L2 larva imaged using the same exposure time (arrow: binucleate tail-spike cell; inset: GFP only). Notice the decreased GFP fluorescence in the tail-spike cell of the *pal-1* mutant. (**C**) Image of a distinctive tail-spike cell corpse (green and inset) persisting in a *ced-5*(*n1812*); *nsIs25* L1 larva. Anterior is to the left. (**D**) *pal-1* is required for tail-spike cell death and acts in parallel to *egl-1*. For each genotype, between 20-52 L1 animals were scored for tail-spike cell corpses, as identified by position, shape and expression of the *nsIs25 C. briggsae ced-3* promoter::GFP transgene. Error, s.e.m. Alleles used: *ced-5*(*n1812*), *pal-1*(*ns114*, *ns115*), *egl-1*(*n1084n3082*). Scale bars: 10 μm.

homeodomain transcription factor *pal-1*. Previously isolated *pal-1* mutants displayed 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 (Fig. 5A). Consistent with these results, we identified a C to T alteration at position 1944 of the *pal-1* genomic sequence in *ns114* animals (Fig. 5B). This mutation creates an ectopic consensus splice-donor site (Fig. 5C) 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). Unlike *ns114*, *pal-1*-null alleles result in fully penetrant lethality accompanied by severe defects in posterior patterning (Edgar et al., 2001), suggesting that the ectopic splice donor site in *ns114* animals may be used only occasionally. In *pal-1*(*ok690*)-null mutants, no detected *ced-3* expression in posterior regions was ever detected (0%, *n*=30); however, we could not reliably identify the tail-spike cells in these animals because of the patterning defects that they displayed.

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 (Fig. 4D). We also mapped *ns115*, and identified a G to A missense mutation at position 1655 of the *pal-1* genomic sequence (Fig. 5B) in this mutant. This mutation results in a glycine to glutamic acid substitution, altering a conserved residue located immediately outside the PAL-1 homeodomain (Fig. 5D). The mild defects in tail morphology observed in *ns115* mutants (data not shown) suggest that *ns115*, like *ns114*, is a weak, or cell-specific, allele of *pal-1*.

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

pal-1 is expressed in a number of posterior cells, including in 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 the PAL-1 protein may directly bind to the *ced-3* promoter to allow *ced-3* expression in the tail-spike cell. Although 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/Cdx2, 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 that we established as being crucial for tail-spike cell *ced-3* expression and death (sites B and C; Fig. 3A). We therefore tested the ability of PAL-1 to bind these promoter sites in an electrophoretic mobility-shift assay. As shown in Fig. 6, a fusion protein between glutathione S-transferase (GST) and the PAL-1 homeodomain (residues 203-270) bound ^{32}P-labeled 22 bp oligonucleotides from the *ced-3* promoter containing either sites B or C (Fig. 6A or 6B, respectively). 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). Furthermore, wild-type oligonucleotides derived from site C competed efficiently with labeled site B oligonucleotides; however, PAL-1-binding-site mutant C oligonucleotides competed less efficiently (Fig. 6C). 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-labeled mutant B or C oligonucleotides (Fig. 6A,B). Additionally, when identical mutations were created in the *ced-3* promoter::GFP reporter, expression in the tail-spike cell was greatly

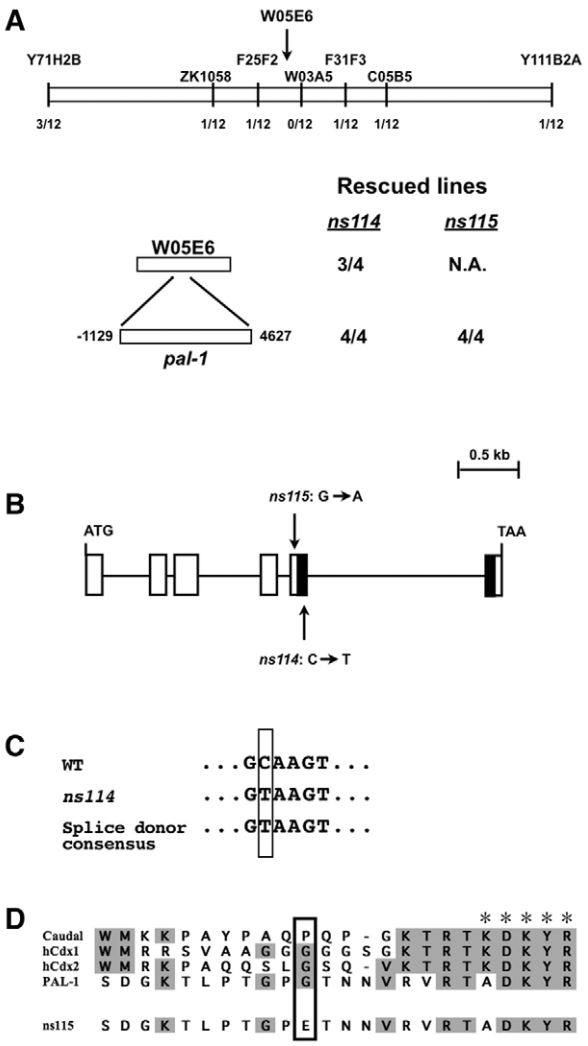

Fig. 5. *ns114* and *ns115* are alleles of *pal-1*. (**A**) Mapping and rescue of *ns114*. Top: *ns114*/CB4856 heterozygotes were allowed to self-fertilize, and homozygous *ns114* progeny were tested for the presence of CB4856 single nucleotide polymorphisms (SNPs). SNP locations are indicated by cosmid names above the rectangle depicting chromosome III, as is the location of the cosmid containing *pal-1*. The number of chromosomes containing CB4856-specific SNPs out of the total examined is indicated under each SNP. Bottom: the number of rescued lines out of the total number of transgenic lines examined is indicated; injections were performed into *pal-1*(*ns114* or *ns115*); *ced-3*(*n717*); *nsIs25* mutants. Details of the rescue experiment are described in the text and in the Materials and methods section. (**B**) 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. (**C**) Comparison of the wild-type and *ns114* mutant site to the consensus *C. elegans* splice donor sequence. Boxed region highlights the mutation. (**D**) Alignment between PAL-1 and its *Drosophila* and human homologs, Caudal and Cdx1/Cdx2. Conserved residues are shaded; asterisks indicate residues comprising the homeodomain; residue mutated by the *ns115* mutation is boxed.

compromised. For example, in four 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 four 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.

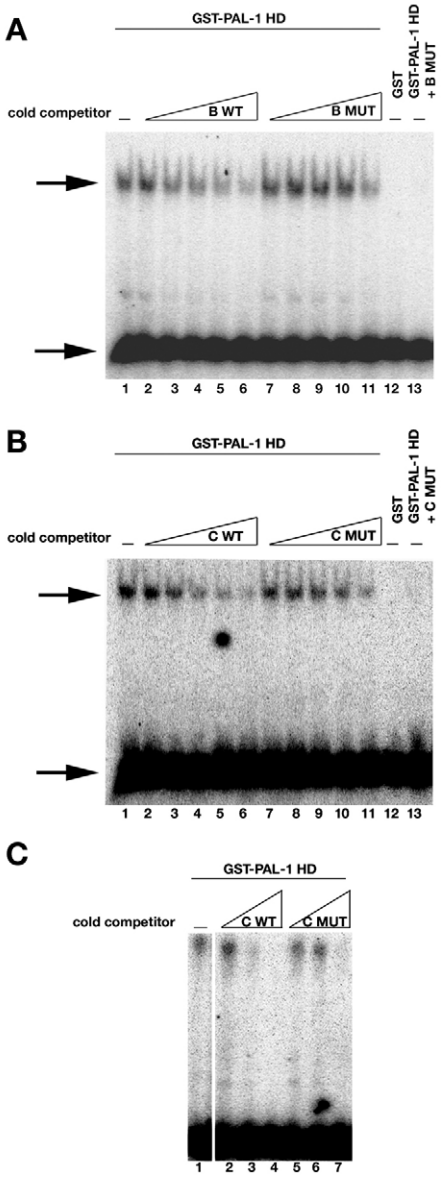

Fig. 6. PAL-1 homeodomain binds to *ced-3* promoter sequences. Electrophoretic mobility shift assay (EMSA) showing binding of labeled DNA derived from sites B and C (**A** and **B**, respectively) of the conserved *C. elegans ced-3* promoter. Cold competitor DNA was added at concentrations of 10× (lanes 2, 7), 100× (lanes 3, 8), 200× (lanes 4, 9), 500× (lanes 5, 10) and 1000× (lanes 6, 11), as indicated by triangles above the autoradiogram. Lane 12 shows no binding of GST to labeled B or C DNA; lane 13 shows absent/weak binding of PAL-1 HD to labeled mutant oligonucleotide. Positions of shifted PAL-1 homeodomain-DNA complex and unbound DNA are indicated with arrows. (**C**) EMSA showing that wild-type DNA derived from site C can compete with labeled DNA derived from site B more effectively than mutant site C DNA. Cold competitor DNA was added at concentrations of 10× (lanes 2, 5), 100× (lanes 3, 6) and 1000× (lanes 4, 7). Details of the assay can be found in the Materials and methods section.

Taken together, these results support the idea that the 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; data not shown), suggesting that this site may bind yet another factor required for the induction of *ced-3* expression in the tail-spike cell.

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 minor roles 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 shortly before the cell displays obvious signs of death, suggesting that regulation of tail-spike cell death may be achieved via the 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 the 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, via interactions with CED-4, leading to the rapid killing of the cell (Fig. 7). 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, because *ced-4* is still absolutely 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 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 (see Results). Similarly, *pal-1* functions independently of *egl-1* (Fig. 4). In addition, we failed to detect expression in the tail-spike cell of two *egl-1* promoter::GFP reporter transgenes, consistent with the idea that *egl-1* plays a minor role in tail-spike cell death.

The time-estimates for *ced-3* transcriptional induction described in the Results section do not account for GFP folding rates; however, several observations suggest that GFP folds rapidly. GFP folds in vitro within 2 minutes (Enoki et al., 2004; Merkel and Regan, 2000) and GFP expression is observed in cultured cells that have been microinjected with a GFP reporter plasmid within 30 minutes of injection (Schmoranzer et al., 2003). To define an upper limit for GFP folding rates in *C. elegans*, we engineered animals containing a heat-shock promoter::GFP transgene, subjected them to a 20-minute heat shock at 34°C and observed robust GFP expression within 30 minutes following heat shock (A. Valentine and S.S., unpublished). Thus, it takes at most 50 minutes for animals to sense increased heat, transcribe and translate GFP, and properly fold the protein. These observations suggest that *ced-3* transcription is induced at a maximum of 80 minutes before the onset of tail-spike cell death. Given that we scored the onset of *ced-3* promoter::GFP expression when the signal is barely detectable and that the signal increases several

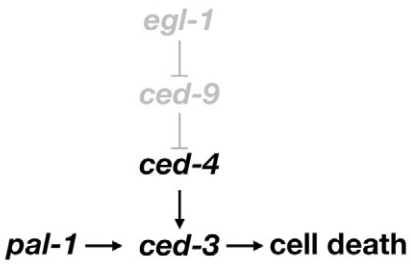

Fig. 7. Model for the regulation of tail-spike cell death. EGL-1 and CED-9 (gray) play minor roles in regulating tail-spike cell death, whereas PAL-1-mediated transcription of *ced-3* is a key regulatory module.

fold over the ensuing 30 minutes, we estimate that the true amount of time between *ced-3* expression and the onset of cell death is significantly less than 80 minutes.

Although our data clearly demonstrate transcriptional control of *ced-3* during tail-spike cell death, *ced-4* transcription may be either constitutive or be 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 use 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 the PAL-1 protein in controlling *ced-3* expression in the tail-spike cell. However, PAL-1 is unlikely to act 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 notice 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 the 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 the 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, in *ced-9*(lf); *ced-3*(weak lf) double mutants, some cells destined to die do so appropriately (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? Transcriptional control of *ced-3* is unlikely to be the main timing mechanism in these cells, because *ced-3* may not be transcribed within dying cells, but rather, 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 a model in which asymmetric segregation/activation of CED-3 protein (or mRNA), which may occur in most *C. elegans* cells fated to die, and transcriptional upregulation of *ced-3* in the tail-spike cell, are the key rate-limiting steps for cell death initiation.

The *ced-9*(*n1950*) gain-of-function allele does not only block the association of CED-9 and EGL-1

Our studies have demonstrated that *egl-1* and *ced-9* behave differently in the tail-spike cell as compared with other cells destined to die in two ways. First, we found that *egl-1*(lf) mutations blocked tail-spike cell death in 30% of the animals examined, whereas *ced-9*(*1950*gf) mutations did not block tail-spike cell death at all. 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). Alternatively, the death-promoting function of *ced-9* may be mediated by its role in mitochondrial fragmentation (Jagasia et al., 2005).

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*) 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, because 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, or due to a role in mitochondrial fragmentation) 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), although 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 the level of Cdx2 expression (Ee et al., 1995; Hinoi et al., 2001). Furthermore, in the intestinal epithelium, Cdx2 is expressed at only 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*.

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Supplementary material

Supplementary material for this article is available at
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